

A Thesis Submitted for the Degree of PhD at the University of Warwick

Permanent WRAP URL:

<http://wrap.warwick.ac.uk/99025>

Copyright and reuse:

This thesis is made available online and is protected by original copyright.

Please scroll down to view the document itself.

Please refer to the repository record for this item for information to help you to cite it.

Our policy information is available from the repository home page.

For more information, please contact the WRAP Team at: wrap@warwick.ac.uk

TRANSLATIONAL CONTROL OF THE HEAT SHOCK
RESPONSE IN XENOPUS OOCYTES

Judith Ann Horrell
(BA Hons., University of Cambridge)

Submitted for the degree of PhD

Department of Biological Sciences
University of Warwick

June 1988

CONTENTS

| | page |
|------------------|------|
| Contents | i |
| List of Figures | vii |
| Acknowledgements | ix |
| Declaration | x |
| Summary | xi |
| Abbreviations | xii |

CHAPTER I: INTRODUCTION

| | |
|---|----|
| I.A Translational control in early development | 1 |
| I.A.i Maternal messenger RNAs | 1 |
| I.A.ii Maternal mRNA in <u>Xenopus</u> | 3 |
| I.A.iii Mechanisms of translational control | 7 |
| I.B The heat shock response | 16 |
| I.B.i Heat shock proteins and control of their synthesis | 16 |
| I.B.ii Translational control of the heat shock response | 22 |
| I.B.iii Different means to the same end | 28 |
| I.B.iv Developmental regulation of hsp expression | 29 |
| I.B.v The heat shock response in <u>Xenopus</u> | 31 |
| I.C The work in this thesis | 37 |
| I.C.i Aim and strategy | 37 |
| I.C.ii The work in this thesis | 39 |

CHAPTER II: MATERIALS AND METHODS

| | |
|----------------------------|----|
| II.A Materials | 43 |
| II.A.i Sources of reagents | 43 |

| | |
|--|----|
| II.A.ii Preparation of buffers,media, and standard reagents | 45 |
| II.B Agarose gel electrophoresis of DNA | 46 |
| II.B.i Agarose gels | 46 |
| II.B.ii Purification of DNA from agarose gels | 48 |
| II.C Use of DNA modification enzymes | 49 |
| II.C.i Restriction enzyme digestion | 48 |
| II.C.ii Treatment with Klenow | 50 |
| II.C.iii Phosphatase treatment | 51 |
| II.C.iv Ligation | 51 |
| II.D Transformations and preparation of plasmid DNA | 51 |
| II.D.i Transformations | 51 |
| II.D.ii Mini plasmid preps | 52 |
| II.D.iii Large-scale plasmid preparation | 53 |
| II.D.iv Caesium chloride density gradients | 55 |
| II.E DNA sequencing | 56 |
| II.E.i Sequencing single-stranded M13 DNA | 56 |
| II.E.ii Sequencing double-stranded plasmid DNA | 59 |
| II.E.iii Sequencing gels | 59 |
| II.F Use of oligonucleotides | 60 |
| II.F.i Work up | 60 |
| II.F.ii Treatment with polynucleotide kinase | 61 |
| II.F.iii Ligation | 61 |
| II.G Oligonucleotide-directed mutagenesis | 62 |
| II.G.i Synthesis of mutant second strand | 62 |
| II.G.ii Transformations | 63 |
| II.G.iii Screening of plaques | 64 |
| II.H Extraction of RNA | 65 |
| II.H.i Extraction of RNA from oocytes | 65 |
| I.H.ii Extraction of influenza virus RNA | 66 |

| | |
|---|----|
| II. I <u>In vitro</u> transcription | 67 |
| II. I. i Synthesis and processing of RNA | 67 |
| II. I. ii Synthesis of RNA probes | 69 |
| II. I. iii Determination of incorporation | 70 |
| II. J Analysis of RNA | 71 |
| II. J. i Selection of poly(A)+ RNA | 71 |
| II. J. ii Formaldehyde agarose gels | 72 |
| II. J. iii Northern blots | 73 |
| II. J. iv RNase mapping | 75 |
| II. K <u>In vitro</u> translation and analysis of proteins | 76 |
| II. K. i <u>In vitro</u> translation | 76 |
| II. K. ii Determination of incorporation of radioactive amino acid | 78 |
| II. K. iii Immunoprecipitation | 78 |
| II. L Electrophoresis of proteins | 79 |
| II. L. i One-dimensional SDS-PAGE | 79 |
| II. L. ii Two-dimensional gel electrophoresis | 82 |
| II. M Microinjection and analysis of oocytes | 84 |
| II. M. i Preparation and microinjection of oocytes | 84 |
| II. M. ii Incubation and processing of oocytes | 85 |
| II. M. iii Estimation of follicle cell numbers | 86 |
| II. M. iv Defolliculation | 86 |

CHAPTER III: RESULTS AND DISCUSSION

SYNTHESIS OF hsp70 IN OOCYTES AND FOLLICLE CELLS

| | |
|---|----|
| III. A Introduction | 88 |
| III. B Defolliculation procedure | 89 |
| III. C hsp70 RNA content of oocytes and follicle cells | 90 |
| III. D Patterns of proteins synthesis in folliculated and defolliculated oocytes | 93 |

| | |
|---|-----|
| III.D.i Folliculated oocytes | 94 |
| III.D.ii Oocytes defolliculated after labelling | 95 |
| III.D.iii Oocytes defolliculated before labelling | 96 |
| III.E Effect of partial removal of follicle cells on protein synthesis | 97 |
| III.F Unusual type of heat shock response | 100 |
| III.G Discussion | 102 |

CHAPTER IV: RESULTS AND DISCUSSION

TRANSCRIPTION OF hsp70 GENES IN XENOPUS OOCYTES

| | |
|--|-----|
| IV.A Introduction | 105 |
| IV.B hsp70 genes | 106 |
| IV.C Construction of an hsp70/globin fusion gene | 107 |
| IV.D Transcription of injected hsp70A and hsp70B genes in oocytes | 108 |
| IV.D.i Rate of transcription | 109 |
| IV.D.ii Location of transcripts | 110 |
| IV.E Sizes of the transcripts from injected genes | 111 |
| IV.F Polyadenylation status of transcripts | 113 |
| IV.G Discussion | 115 |

CHAPTER V: RESULTS AND DISCUSSION

EXPRESSION OF hsp70 FROM INJECTED GENES

| | |
|---|-----|
| V.A Introduction | 117 |
| V.B Expression of hsp70/globin fusion protein | 118 |
| V.C Expression of hsp70A | 119 |
| V.D Self-degradation of hsp70 | 121 |
| V.E Expression of hsp70 during unusual heat shock response | 122 |
| V.F Discussion | 123 |

CHAPTER VI: RESULTS AND DISCUSSION

TRANSLATION OF SYNTHETIC hsp70 RNA IN OOCYTES

| | |
|---|-----|
| VI.A Introduction | 125 |
| VI.B Subcloning of native and mutant hsp70 sequences into pGEM-2 | 126 |
| VI.B.i pGEM270B/A | 127 |
| VI.B.ii pGEM270B/G | 128 |
| VI.B.iii pGEM270B/X | 128 |
| VI.B.iv <u>In vitro</u> and <u>in vivo</u> translation | 130 |
| VI.C Subcloning of mutant hsp70 sequences into pSP64T | 131 |
| VI.D Site-directed mutagenesis | 132 |
| VI.E Subcloning of hsp70* sequences into pGEM-1 | 136 |
| VI.F Subcloning into pSP64T and expression in oocytes of hsp70B/A, hsp70A* and hsp70B* | 137 |
| VI.F.i Subcloning | 137 |
| VI.F.ii Expression | 138 |
| VI.G Discussion | 139 |

CHAPTER VII: RESULTS AND DISCUSSION

EXPRESSION OF PREPROCHYMOSIN AND FLU NUCLEOPROTEIN

DURING HEAT SHOCK

| | |
|--|-----|
| VII.A Introduction | 140 |
| VII.B Expression of NP during heat shock | 141 |
| VII.B.i Patterns of NP expression | 141 |
| VII.B.ii Effect of DNA concentration | 145 |
| VII.C Expression of chymosin during heat shock | 146 |
| VII.D Coinjection of pTKNP and pTK82 | 148 |
| VII.E Discussion | 148 |
| VII.F Insertion of heat shock oligonucleotide into | |

| | |
|--------------------------------------|-----|
| pTK82 and expression in oocytes | 150 |
| VII.F.i Insertion of oligonucleotide | 150 |
| VII.F.ii Expression | 151 |
| VII.G Translation of injected RNA | 152 |

CHAPTER VIII: CONCLUSIONS AND PROSPECTS

| | |
|---|-----|
| VIII.A Introduction | 154 |
| VIII.B The nature of the heat shock response | 154 |
| VIII.C Effects of heat shock on translation of exogenous transcripts | 158 |
| VIII.D Oocytes as a system for investigating translational control | 160 |

| | |
|------------------------|-----|
| CHAPTER IX: REFERENCES | 163 |
|------------------------|-----|

| | |
|----------|-----|
| APPENDIX | 181 |
|----------|-----|

Reprint of:

Horrell, A., Shuttleworth, J., and Colman, A. (1987).
Transcript levels and translational control of hsp70
synthesis in Xenopus oocytes. Genes Dev. 1, 433-444.

LIST OF FIGURES

| | |
|--|-----|
| Fig. III.1 Oocytes at various stages of defolliculation | 89 |
| Fig. III.2 Levels of hsp70A and hsp70B transcripts in oocytes and follicle cells | 91 |
| Fig. III.3 Normal patterns of protein synthesis at 20°C and during heat shock | 94 |
| Fig. III.4 Two-dimensional analysis of protein synthesis in oocytes with and without follicle cells attached | 96 |
| Fig. III.5 Partial defolliculation of oocytes | 98 |
| Fig. III.6 Unusual pattern of protein synthesis | 100 |
| Fig. IV.1a The 5' sequence of genomic hsp70A and hsp70B clones | 106 |
| Fig. IV.1b Restriction sites in phsp70A and phsp70B | 106 |
| Fig. IV.2 Construction of phsp70B/G | 107 |
| Fig. IV.3 <u>In vitro</u> translation of hsp70B/A and hsp70B/G SP6 mRNA | 108 |
| Fig. IV.4 Transcription from injected hsp70 genes | 109 |
| Fig. IV.5 Accumulation and location of RNA transcribed from injected hsp70A genes | 110 |
| Fig. IV.6a Northern blot showing hsp70 transcripts in follicle cells and injected oocytes | 112 |
| Fig. IV.6b Northern blot analysis of <u>X. borealis</u> oocyte RNA | 112 |
| Fig. IV.7a Poly(A) status of hsp70 transcripts | 113 |
| Fig. IV.7b Northern blot of oviduct poly(A)+ RNA selected by oligo-dT cellulose | 113 |
| Fig. IV.8 Repeated oligo-dT selection of hsp70 mRNA | 114 |

| | |
|--|-----|
| Fig. V.1 Expression of hsp70B/G in oocytes | 119 |
| Fig. V.2 Expression of hsp70 in oocytes | 120 |
| Fig. V.3 Self-degradation of hsp70 | 121 |
| Fig. V.4 Expression of hsp70A in oocytes exhibiting unusual heat shock response | 122 |
| Fig. VI.1a Construction of pGEM270B/A | 127 |
| Fig. VI.1b Construction of pGEM270B/G | 128 |
| Fig. VI.1c Construction of pGEM270B/X | 129 |
| Fig. VI.2 Construction of pSP64T70B/G and pSP64T70B/X | 131 |
| Fig. VI.3 <u>In vitro</u> translation of synthetic RNAs | 132 |
| Fig. VI.4 <u>In vitro</u> mutagenesis | 134 |
| Fig. VI.5 Sequence of pGEM170A* and pGEM170B* | 136 |
| Fig. VI.6 Transfer of hsp70A* and hsp70B* into pSP64T | 137 |
| Fig. VI.7 Injection of SP64T-derived hsp70 RNA into oocytes | 137 |
| Fig. VI.8 Structures of synthetic hsp70 RNAs | 139 |
| Fig. VII.1 Expression of pTKNP' during heat shock in oocytes | 141 |
| Fig. VII.2 Different patterns of pTKP expression | 143 |
| Fig. VII.3 Effect on pTKNP expression of amount of DNA injected | 145 |
| Fig. VII.4 Expression of pTK82 and stability of preprochymosin in oocytes | 146 |
| Fig. VII.5 Coinjection of pTK82 and pTKNP | 148 |
| Fig. VII.6 Construction of pTKHS+ and pTKHS- | 150 |
| Fig. VII.7 Translation of injected mRNA in oocytes during heat shock | 152 |

ACKNOWLEDGEMENTS

I am grateful to my supervisor Alan Colman for his advice, encouragement and practical help throughout the project, and for the extended loan of his computer during the apparently interminable writing-up period. John Shuttleworth, with whom I worked closely during a large part of the project, gave me much essential advice and training on the methods, and was involved in many long discussions about results.

Valuable assistance and advice was also provided by other members of the Animal Molecular Genetics Group. They are too numerous to thank individually, but I am particularly grateful to Douglas Drummond, Glenn Matthews, Rachel Strachan, Linda Tabe, Gareth Cross, Dave Jackson, Fiona Aldridge and Surinder Bhamra, and to Christine Newton and Ed Bagenal for keeping the lab running smoothly.

Financial support was provided by the Science and Engineering Research Council.

DECLARATION

I declare that the work reported in this thesis was carried out by myself, except where the collaboration or assistance of others is acknowledged in the text. This thesis has not previously been submitted for any degree.

The major part of Chapter III and sections of Chapters IV and V have been published as:

Horrell, A., Shuttleworth, J., and Colman, A.
(1987). Transcript levels and translational
control of hsp70 synthesis in Xenopus oocytes.
Genes Dev. **1**, 433-444.

SUMMARY

It has been suggested that the heat shock response of *Xenopus* oocytes might provide a good model system to study the regulation of translation in early development: oocytes were thought to contain a store of hsp70 mRNA, which was unmasked reversibly on heat shock. However, more recently it has been reported that the hsp70 apparently synthesized in oocytes is in fact made entirely in the attached follicle cells.

A method was developed to reliably remove follicle cells. Two-dimensional gel analysis of radioactively labelled oocytes revealed that, although hsp70 was the major protein synthesized during heat shock if follicle cells were present, it could not be detected if these cells were removed. Oocytes do contain up to 15pg of hsp70 mRNA; it is not clear if this is translated. In some experiments synthesis of a group of proteins was induced in oocytes during heat shock, but none was hsp70.

To test whether oocytes can synthesize hsp70, plasmids encoding *Xenopus* hsp70 genes were injected. These were transcribed efficiently, yielding RNA that was indistinguishable in length and polyadenylation status from heat-induced transcripts in follicle cells. Synthesis of hsp70 was detected in defolliculated, injected oocytes: it was translated well even during heat shock (when translation of other mRNAs was greatly reduced), suggesting that oocytes regulate translation of exogenous transcripts appropriately. Surprisingly, flu nucleoprotein RNA transcribed in oocytes was also preferentially translated during heat shock in some experiments. The 5' leaders of hsp70 and NP RNAs contain a region of homology.

As injection of RNA provides a more direct way of controlling transcript levels in oocytes, polyadenylated RNA encoding hsp70 was transcribed *in vitro* (from a variety of templates) and injected into oocytes. Products of a hsp70/globin fusion and a truncated hsp70 gene were never detected. Native hsp70 was synthesized, but it was not made preferentially during heat shock. Similarly, when influenza NP mRNA was injected, preferential translation was never observed. It appears therefore that exogenous RNA is regulated appropriately during heat shock when it is introduced by the route of DNA injection, but not when RNA itself is injected.

ABBREVIATIONS

| | |
|---------------|--|
| Bisacrylamide | N,N'-methylene bisacrylamide |
| BSA | bovine serum albumin |
| CIP | calf intestinal phosphatase |
| DMF | dimethyl formamide |
| DTT | dithiothreitol |
| HEPES | N-2-Hydroxyethylpiperazine-N'- 2-ethanesulphonic acid |
| hsp | heat shock protein |
| IPTG | isopropyl- β -D-thio-galactopyranoside |
| MBS | modified Barthe's saline |
| MOPS | 3-[N-morpholino]-propanesulphonic acid |
| PIPES | piperazine-N,N'-bis[2-ethanesulphonic acid] |
| PMSF | phenylmethylsulphonylfluoride |
| RNasin | human placental RNase inhibitor |
| SDS | sodium dodecylsulphate |
| SDS-PAGE | sodium dodecylsulphate-polyacrylamide gel electrophoresis |
| TCA | trichloroacetic acid |
| TEMED | N,N,N',N'-tetramethylethylenediamine |
| TK | thymidine kinase |
| X-gal | 5-bromo-4-chloro-3-indolyl- β -galactoside |

CHAPTER I: INTRODUCTION

I.A TRANSLATIONAL CONTROL IN EARLY DEVELOPMENT

The involvement of translational regulation in early development poses some of the major questions in developmental biology. For several hours after fertilization, the embryonic genome is inactive. All protein synthesis occurring during this time is therefore directed by mRNA made in the oocyte, which is translated only at low levels before fertilization. How is this increase in protein synthesis rate controlled? And what different or additional mechanisms operate on the relatively few RNA species whose recruitment is either delayed or much more rapid than that of the bulk of the maternal message stockpile?

I.A.i Maternal messenger RNAs

During oogenesis in all animals, a store of macromolecules is accumulated, to provide proteins needed in early development, and particularly before transcription from the embryonic genome is activated (reviewed by Smith and Richter, 1985, and Davidson, 1986). In *Xenopus*, this activation does not occur until the mid-blastula transition (MBT) about eight hours after fertilization, when the embryo already contains around 4000 cells (Newport and Kirschner, 1982). In other organisms, embryonic transcription is activated after fewer cycles, but because the cycles are slower, the timescale is similar: for example, in the mouse, transcription is greatly stimulated at the two-cell stage, 16-20 hours after fertilization (Davidson, 1986).

As well as proteins themselves, the store contains messenger RNAs and components of the translational machinery.

This maternal mRNA is indispensable for development - as was demonstrated by the early discoveries that sea urchin eggs can divide when transcription is inhibited with actinomycin D (Brachet and Denis, 1963), but not when translation is inhibited with puromycin (Hultin, 1961a). On fertilization, there is an increase in the rate of protein synthesis as this previously untranslated RNA is recruited onto polysomes. This is most dramatic in sea urchin eggs: the rate of protein synthesis in unfertilized eggs is very low, and increases a hundred-fold on fertilization (Hultin, 1961b). In *Xenopus*, a more modest two-fold increase is observed, but it occurs when meiosis resumes at maturation, rather than at fertilization (Wasserman *et al.* 1982). The difference in timing is probably related to the fact that sea urchins store mature haploid eggs, whereas in frogs hormonally induced oocyte maturation occurs immediately before the eggs are laid.

In addition to the global changes in rates of protein synthesis, there are some spectacular examples of specific translational regulation of certain mRNAs. In the sea urchin and clam, for example, fertilization triggers an increase in the translation of several major maternal mRNAs much greater than the overall increase in protein synthesis rate, and causes a decrease in the translation of others (Rosenthal *et al.* 1980; Evans *et al.* 1983). One of the major new proteins, cyclin, is synthesized continuously during cleavage, but broken down at a particular point in each division cycle. Another of the newly synthesized polypeptides in clams is the small subunit of ribonucleotide reductase (Standart *et al.* 1985). In contrast, the large subunit is one of the major polypeptides in the oocytes, and its level does not increase

during cleavage (Standart et al. 1986). This enzyme thus illustrates the two extremes in the strategies adopted to provide early embryos with sufficient protein. Differential regulation of the translation of maternal mRNA species also occurs in Xenopus, and is discussed in detail below (I.A.ii).

So, maternal mRNA is indispensable to the early development of all animals. The exact mode of utilization is adapted to the needs of particular organisms, but there are many features in common. In particular, the translational control of this mRNA is crucial. It has two components - the regulation of the global increase in the rate of protein synthesis, and the control of the differential recruitment of specific mRNAs at particular developmental stages.

I.A.ii Maternal mRNA in Xenopus

Overall rate of protein synthesis: Stage 6 Xenopus oocytes contain about 90ng poly(A)+ RNA (Dolecki and Smith, 1979). However, of this, an estimated 55ng contains interspersed repeat sequences (Anderson et al. 1982; Richter et al. 1984). This does not compete with globin mRNA for translation when injected into oocytes, and is very poorly translatable in vitro. Since oocytes do contain enough bona fide mRNA to account for translation up to the MBT (see below), Richter et al (1984) conclude that the interspersed RNA may never be processed to become translatable. Its role is unresolved.

Fully grown oocytes also contain 21ng putative non-mitochondrial mRNA, and all species of this RNA have reached steady state levels by stage 2 of oogenesis (Golden et al. 1980): transcription occurring after stage 2 replaces RNA lost by turnover.

Taylor and Smith (1985) have demonstrated that the absolute rate of protein synthesis increases by more than 100-fold between stages 1 and 6 of oogenesis, although, as the number of ribosomes increases by the same amount, the proportion of ribosomes in polysomes remains constant at around 2% (this confirms the results of Woodland, 1974). The ribosome packing density and transit time also stay constant, so Taylor and Smith conclude that the increase in rate of protein synthesis must be accounted for by the translation of more mRNA molecules, and estimate that 0.8ng mRNA is in polysomes at stage 3 and 4ng (i.e. almost 20%) at stage 6. Since enucleation of stage 6 oocytes does not affect the rate of protein synthesis, and few, if any, of the newly synthesized cytoplasmic transcripts are found on polysomes (Dolski and Smith, 1976), it seems that newly translated mRNA molecules must be recruited from the maternal stockpile, not simply provided by de novo synthesis.

The two-fold increase in rate of protein synthesis on maturation (Wasserman et al. 1982) is followed by a further increase of about 1.5-fold at fertilisation, and by the blastula stage about 15% of ribosomes are in polysomes (Woodland, 1974). No transcription occurs during this period, and ribosome transit times and the size of polysomes remain constant (Richter et al. 1982), so the increase in protein synthesis must again be due to recruitment of the maternal stockpile. The estimated 17ng untranslated mRNA in stage 6 oocytes is probably just sufficient to account for this, without the need for interspersed poly(A)+ RNA to be converted to a translatable form (Richter et al. 1984).

Specific mRNA species: Several studies have demonstrated that most mRNA species are in both the translated and the untranslated fractions in the oocyte, so that when the untranslated RNA is recruited, the overall pattern of synthesis does not change greatly (Ballantine *et al.* 1979; Wasserman *et al.* 1982). Ballantine *et al.* (1979) analysed newly synthesized proteins in oocytes, matured oocytes, and at various stages during early development on two-dimensional SDS-polyacrylamide gels. They could detect the appearance of no new proteins before the end of the blastula stage, although in a similar study Wasserman *et al.* (1982) did find one protein that appeared after progesterone-induced maturation.

However, there are examples of mRNA species that are differentially regulated during oogenesis and early development. Ballantine *et al.* reported that the synthesis of several proteins, including β - and γ -actin, was reduced on maturation and fertilization. These proteins began to be synthesized again during cleavage. In contrast, in their studies on maturation, Wasserman *et al.* did not observe this effect, and suggest that the differences may be due to the fact that Ballantine *et al.* loaded equal incorporated cpm onto their gels: as the overall rate of protein synthesis increases on maturation/fertilization, less total protein would have been loaded for the egg and embryo samples than for the oocyte samples. The disappearance of certain spots may therefore reflect delayed recruitment of the corresponding mRNAs, rather than an actual reduction in synthesis in the egg.

Other examples of delayed recruitment have been reported. Dworkin *et al.* (1985) randomly selected 18 moderately prevalent sequences from an ovary cDNA library, and used them

to probe polysomal and non-polysomal RNA. Most of the sequences are largely non-polysomal in stage 8 oocytes, and 13 are almost entirely polysomal by the 16-cell stage. Four sequences, however, do not accumulate in polysomes until after this stage, and in at least one case this does not occur until gastrulation. It is not known whether the RNA recruited then is maternal or newly transcribed from the embryonic genome. Dworkin et al also identified three other mRNA species that decrease in abundance during oogenesis, and are poorly recruited after fertilization. These encode enolase, the ADP-ATP carrier protein, and α -tubulin.

It has also been reported that the synthesis of fibronectin and the nuclear lamins L₁ and L₂ increases dramatically around the MBT (Lee et al, 1984; Stick and Hausen, 1985). As inhibition of transcription with α -amanitin does not prevent any of these increases, they must be due at least in part to further recruitment of maternal message. The control of histone synthesis provides further notable examples of differential translational regulation (reviewed by Woodland, 1980). There is a 50-fold increase in the rate of core histone synthesis at maturation, when there is only a two-fold increase in overall protein synthesis. By contrast, the recruitment of H1 mRNA is delayed until the mid-blastula transition .

Selective translation also occurs during oogenesis. For example, by stage 2 of oocyte development, 50% of mRNA encoding ribosomal proteins is found on polysomes, compared with only 2% of the bulk mRNA (Pierandrei-Amaldi et al, 1982; Baum and Wormington, 1985). The ribosomal proteins continue to be synthesized at these high levels throughout oogenesis, but,

as the overall rate of protein synthesis increases by five- to eight-fold over this period, their contribution as a proportion of total synthesis decreases.

I.A.iii Mechanisms of translational control

The utilization of maternal mRNA in early development therefore provides some of the best documented examples of translational control (see Davidson, 1986) and it has consequently been the subject of intensive investigation. A variety of control mechanisms have been postulated, which act both on the translational machinery (by modifications to the ribosomes or activation of translation factors) and on the mRNAs themselves. These latter include the addition and removal of 'masking' proteins, structural modification, and transfer of mRNA species between different cellular compartments. In the following discussion of these mechanisms, I draw on examples from Xenopus where possible, but also include relevant examples from other systems.

Modifications to translational apparatus: Although the number of ribosomes is not limiting in oogenesis in Xenopus (see I.A.ii), it is theoretically possible that the gross changes in protein synthesis rates could be controlled entirely by a modification to the ribosomes or the supply of some other component of the translational apparatus. Practical evidence for this was obtained by Laskey et al (1977), who demonstrated that the translational capacity of stage 8 oocytes is limiting. Injected globin mRNA is translated only at the expense of endogenous mRNAs. Recently, however, Taylor et al (1985a) extended this study to growing oocytes, and found that

these do have spare translational capacity. Translation must therefore be limited by the availability of mRNA - at these early stages at least.

Although limiting translational capacity cannot account for the masking of mRNA throughout oogenesis, the capacity must none the less increase on maturation or fertilization. The situation in sea urchin eggs is similar, in that globin mRNA is translated only at the expense of endogenous mRNA (Colin and Hille, 1986). As the ribosomal protein S6 is phosphorylated on fertilization in Arbacia punctulata (Ballinger and Hunt, 1981), a role for it was implicated in the increase in translational capacity which occurs on fertilization. However, it has subsequently been found that some treatments (such as weak bases which mimic the increase in intracellular pH that occurs on fertilization) activate protein synthesis but have no effect on the phosphorylation state of S6 (Ballinger et al. 1984). Moreover, in two other species of sea urchin, S6 is heavily phosphorylated in the unfertilized egg, and it is not affected by fertilisation (Ward et al. 1983), so its exact role is unclear.

The increased intracellular pH associated with fertilisation in sea urchin eggs occurs at maturation in Xenopus oocytes. It is again temporally correlated with phosphorylation of the protein S6, and stimulation of protein synthesis (reviewed by Taylor et al. 1985b). However, the three events can be uncoupled: for example, priming of a frog with pregnant mare's serum gonadotrophin increases the intracellular pH and protein synthesis rate of its oocytes, but has no effect on S6 phosphorylation (Stith and Maller, 1985). When injection of globin mRNA into stage 4 oocytes is

followed by injection of maturation promoting factor, there is a dramatic increase in S6 phosphorylation, but no further increase in protein synthesis (Taylor et al. 1985b). It appears, then, that S6 phosphorylation is neither necessary nor sufficient to account for the increases in protein synthesis seen during oogenesis and early development.

Several recent investigations have suggested that translation factor modification may be involved in control of this increase (see Clemens, 1987). In direct contrast to the results of Laskey et al. (1977), Audet et al. (1987) have found that polysomes compete as effectively as naked mRNA for translation when injected into fully grown Xenopus oocytes, so they concluded that the rate-limiting factor is not associated with the ribosomes. Although Lingrel and Woodland (1974) had previously suggested that initiation is not the rate-limiting step, Audet et al. tested the effects on protein synthesis of injecting eukaryotic initiation factors. Only eIF-4A (one of the three subunits of eIF-4F, which is involved in cap recognition and binding of mRNA to ribosomal subunits) had any effect, and this increased the rate by two-fold. As this is comparable to the increase normally induced by maturation, and subsequent progesterone-induced maturation of injected oocytes does not cause a further increase, it seems possible that eIF-4A is responsible for the stimulation of protein synthesis seen in vivo.

Unfertilized sea urchin eggs contain an inhibitor of translation that inhibits protein synthesis in rabbit reticulocyte lysate (Huang et al. 1987). This can also be reversed by the addition of eIF-4F. However, although addition of eIF-4F to lysates of unfertilized eggs does stimulate

translation, it does not raise the rate to that observed in two-hour embryos. It appears, therefore, that it is not the only mechanism involved. The rate of translation in extracts of sea urchin eggs is also increased by the addition of eIF-2 (responsible for the binding of met-tRNA to the ribosome) or GEF (the guanine-nucleotide exchange factor, which catalyzes the recycling of eIF-2) (Colin et al., 1987; Clemens, 1987). However, this occurs only when exogenous mRNA is present - so these factors cannot activate endogenous masked mRNA. It has been demonstrated that the phosphorylation state of eIF-4E and the α -subunit of eIF-2 are altered by fertilization.

mRNA binding proteins: It has long been speculated that translation of maternal mRNA molecules in oocytes and eggs is prevented by 'masking' proteins, and early results of experiments on sea urchin eggs tended to support this view. Messenger ribonucleoprotein particles (mRNPs) isolated in buffers with ionic composition similar to those in the eggs did not stimulate translation in the wheatgerm in vitro translation system. In contrast, mRNA extracted from them, and mRNPs prepared in non-physiological conditions both stimulated translation in vitro (Jenkins et al., 1978; Ilan and Ilan, 1978). Proteins were identified that were present in mRNPs from eggs, but not in hatched blastulae, and which might therefore be involved in translational control in eggs (Moon et al., 1980).

A subsequent systematic investigation, however, cast doubt on these results. This failed to show a difference of more than two-fold in translatableity in vitro between mRNPs isolated in a variety of different conditions and the mRNA

extracted from them (Moon et al. 1982). More recently, though, Winkler et al. (1985) have produced evidence that revives the 'masked message' hypothesis: they found that, although the translational capacity of sea urchin egg lysate is indeed limited, the mRNPs in the lysate are not translated even when it is mixed with reticulocyte lysate. They are activated by treatment with high salt and EDTA (Winkler et al. 1986), suggesting that unmasking *in vivo* may occur by the removal of proteins. Furthermore, mRNPs partially purified from eggs by gel filtration are inactive, while mRNPs prepared in the same way from fertilized eggs are active (Grainger and Winkler, 1987). However, other mechanisms of limiting mRNA availability may of course also operate, and these are discussed below.

In *Xenopus*, the proteins bound to mRNA in oocytes have been extracted and analysed. Groups of four or five major oocyte-specific proteins have been identified that bind preferentially to poly(A)+ mRNA and decrease in abundance during oogenesis (Darnbrough and Ford, 1981; Richter and Smith, 1983). The synthesis of two of these proteins (of 50kD and 52kD) is greatest at stage 2 and declines as oogenesis continues, although the proteins are still detectable in fully grown oocytes. The synthesis of the two other major proteins (of 56kD and 59kD) decreases sharply after stage 1 and they are not detectable in fully grown oocytes (Dixon and Ford, 1982). A monoclonal antibody to the 56kD protein was used to select a clone from an immature ovary cDNA library, and RNA that hybridized to the clone decreased in abundance during oogenesis, and was not found in somatic cells (Lorenz and Smith, 1985).

All this evidence is consistent with a role for these proteins in the general suppression of translation of mRNA early in oogenesis, and its subsequent gradual unmasking. More direct evidence was obtained by reconstituting globin mRNA in vitro with proteins from the mRNPs of stage 1 and 2 oocytes (Richter and Smith, 1984). The resulting mRNPs were translated very poorly when injected into stage 6 oocytes, although globin synthesis was readily detectable when naked mRNA was injected. The 56kD and 60kD proteins are phosphorylated in vivo (Dearsly et al. 1985), and dephosphorylation of these proteins leads to destabilization of reconstituted globin mRNP complexes, and restores the translatability of the mRNA in both in vitro translation systems and oocytes (Kick et al. 1987).

It seems likely, therefore, that these abundant mRNA binding proteins are involved in the storage and recruitment of bulk mRNA in oocytes. It is also possible that the same basic principle applies to the differential regulation of translation: specific proteins might bind to individual mRNAs or groups of mRNAs, allowing them to be unmasked at particular stages of development.

Sequestration of mRNA: Other mechanisms can be envisaged that would achieve the same end as masking of mRNA by proteins. One such mechanism would be the physical sequestration of the mRNA in a different cellular compartment from the translational apparatus. Again, this mechanism could account for both general and specific examples of translational control.

There is evidence that this might be involved in the regulation of histone synthesis in sea urchin eggs and embryos. Transcripts corresponding to the early histone repeat sequences are concentrated between 25 and 50-fold in the pronuclei of eggs (Venesky et al. 1981), although a significant proportion of histone transcripts is also present in the cytoplasm. The nucleolar transcripts, which do not begin to accumulate until after maturation (Angerer et al. 1984), appear to be authentic histone mRNA, and contain no spacer sequences (DeLeon et al. 1983). They are released into the cytoplasm at nuclear membrane breakdown (NMBD) of the first cleavage division, after which they begin to enter polysomes. However, transcripts enter polysomes even if NMBD is inhibited (Showman et al. 1982). It is not clear whether these newly translated transcripts were already in the cytoplasm, or whether there is a transport mechanism for histone mRNA different from passive release at NMBD.

Message sequestration does remain an attractive possibility. It is apparently not a general mechanism, however, as in situ hybridization using poly(U) as a probe reveals that less than 1% of total poly(A)+ RNA is nuclear, and that there are no major sites of localization in the cytoplasm, at least of bulk poly(A)+ RNA (Angerer and Angerer, 1981). Moreover, three maternal mRNAs that are not translated until fertilization in oams are evenly distributed throughout the cytoplasm of oocytes (Swenson et al. 1987).

Polyadenylation of mRNA: The poly(A) tracts at the 3' end of eukaryotic mRNAs are important for the stability and translatability of the mRNA. For example, synthetic mRNA injected into *Xenopus* oocytes is more stable and translated more efficiently if it is polyadenylated (Kreig and Melton, 1984; Drummond et al. 1985). During development there are changes in the amount of poly(A), and it has been speculated that these are involved in the regulation of translation - although definitive evidence is lacking.

The dynamics of poly(A)-containing RNA are best documented in sea urchin eggs (see for example Wilt, 1977): there is extensive turnover of poly(A) throughout development, and most sequences are represented in both the poly(A)+ and the poly(A)- classes. On fertilization, the average length of poly(A) tails increases from 45 to 80 bases, and the number of molecules with poly(A) tails increases.

In clams, the widespread and sequence-specific changes in polyadenylation patterns that occur on fertilization (Rosenthal et al. 1983; Rosenthal and Ruderman, 1987) are well correlated with translatability. Four classes of mRNA have been identified: (1) those not translated in oocytes but translated with very high efficiency immediately after fertilization; (2) those not translated in oocytes and partially mobilized after fertilisation; (3) those translated in oocytes and not translated after fertilisation; and (4) those not translated detectably before or after fertilisation. (In contrast to *Xenopus*, very few mRNAs are translated both in the oocytes and in the fertilized eggs of the clam *Spisula*.) The poly(A) tails of mRNAs whose translatability increases on fertilisation lengthen, while

those on mRNAs whose translatability decreases shorten.

The conclusion that addition and removal of poly(A) are involved in developmental regulation of translation is supported by results from other systems. The mRNA encoding cyclin in starfish is poly(A)- and untranslated before maturation; on maturation it becomes polyadenylated and its translation is dramatically stimulated (Standart et al. 1987). When development is initiated in Dictyostelium, the rate of protein synthesis falls and newly synthesized mRNA is translated preferentially. This discrimination is accompanied by a rapid shortening of the poly(A) tails on preexisting mRNA (Palatnik et al. 1984). The synthesis of the major poly(A) binding proteins p31 and p31.5 is also dramatically reduced, and preexisting p31 and p31.5 is rapidly degraded (Manrow and Jacobson, 1987).

5' cap methylation: Another change in mRNA structure associated with fertilization (at least in sea urchins) is the increase in methylation of the 5' cap structure (Caldwell and Emerson, 1985). As more than 40% of the newly methylated mRNAs are the early histone-encoding messages previously sequestered in the pronucleus, it seemed likely that the process regulated translational activation of these mRNAs. However, treatment of eggs and embryos with aphidicolin abolishes cap methylation without affecting early histone synthesis (Showman et al. 1987). The role of this modification is unclear, although it has been reported that cap methylation enhances translation in some cell-free systems (see Showman et al. 1987).

1.2 THE HEAT SHOCK RESPONSE

1.2.1 Heat shock proteins and control of their synthesis

When any organism is subjected to an increase above its normal ambient temperature, it responds rapidly by dramatically altering the pattern of gene expression at the levels of both transcription and translation. The result is that expression of most genes is greatly reduced, while synthesis of a set of specialized 'heat shock' proteins (hsps) is induced (reviewed by Schlesinger *et al.* 1982; Lindquist, 1986). These characteristics have made this universal and highly conserved response a very fruitful model system for studying many aspects of the control of gene expression, particularly in eukaryotes.

Heat shock proteins: Many proteins have been reported to be induced by heat shock - including such apparently unlikely examples as enolase in yeast (Iida and Yahara, 1985) and H2B in *Drosophila* (Saunders, 1981). However, the most prominent and consistently observed hsps fall into three groups: 70kD; 83-90kD; and 20-30kD.

The most abundant and highly conserved of the heat shock proteins is hsp70. There is 75% homology between the human and *Drosophila* proteins, and 50% homology between human hsp70 and the *E. coli* equivalent, the *dnaK* product (Hunt and Morimoto, 1985). Some regions of the protein are almost perfectly conserved. Eukaryotic species each contain a group of closely related 70kD proteins. At least one member of the group is heat-inducible (i.e. hsp70), but the remainder ('cognate' proteins, hsc) are expressed constitutively or at particular points in development (Craig *et al.* 1983).

The eight-member yeast hsp70 gene family, for example, contains four subgroups (SSA-SSD) each of which encodes a group of functionally related proteins (Werner-Washburne et al., 1987). Transcription of most SSA genes is induced by heat shock, and their products confer thermotolerance (Craig and Jacobsen, 1984); transcription of SSB genes is decreased by heat shock, their products confer cold tolerance (Craig and Jacobsen, 1985); the SSC gene is transcribed at normal temperatures and is required for normal growth (Craig et al., 1987).

Immunofluorescence studies on the cellular localization of hsp70 in monkey COS cells have demonstrated that it concentrates in the nucleoli during heat shock, but returns to the cytoplasm on recovery (Welch and Feramisco, 1984; Pelham et al., 1984). The interaction with nucleoli can be disrupted in vitro by ATP (Lewis and Pelham, 1985). However, hsp70 also associates with a wide variety of proteins including: heterogeneous nuclear RNPs and messenger RNPs (Kloetzel and Bautz, 1983); the nuclear matrix and cytoskeleton; calmodulin (see Schlesinger et al., 1982; Clark and Brown, 1986); and the transformation-related protein p53 (Hinds et al., 1987). Clathrin-uncoating ATPase has been identified as a member of the hsp70 family on the basis of immunological cross-reactivity and co-migration on two-dimensional gels (Ungewickell, 1985; Chappell et al., 1986).

All eukaryotes produce at least one protein from the second most highly conserved group of hsp's, which are in the range 83-90kD. Unlike hsp70, this high molecular weight hsp is abundant in non-heat-shocked cells in most vertebrates, but is further induced on heat shock (see Lindquist, 1986). It is a

soluble cytoplasmic protein (Lai et al. 1984) and associates with various 'interesting' proteins including retroviral transforming proteins (Operman et al. 1981) and other proteins involved in intracellular signalling (see Lindquist, 1986).

In addition, all organisms produce at least one small (20-30kD) hsp, but these form a very heterogeneous group. Most show extensive homology with α -crystallin, a major component of vertebrate lens (Ingolia and Craig, 1982), which is probably related to their ability to aggregate to form higher order structures (Arrigo and Ahmad-Zadeh, 1981; Arrigo et al. 1985). Like hsp70, the small hsp's are localized in nuclei during heat shock and return to the cytoplasm on recovery (Arrigo, 1987).

Function of hsp's: What do these observations reveal about the function of hsp's? It has been assumed that hsp's somehow prevent and/or repair damage to cellular structures caused by heat shock, and that cognate proteins perform similar roles at normal temperatures. Heat shock causes partial denaturation of proteins, which form insoluble aggregates. Pelham (1986) has proposed that hsp70 disrupts these aggregates - and allows proteins to refold to their native state - by binding to exposed hydrophobic surfaces then using ATP hydrolysis to release itself.

Two very recent studies have provided exciting evidence to support this type of model. Yeast with deletions in SSA1, SSA2 and SSA4 is non-viable, but is rescued by plasmids containing an hsp70 gene under the GAL1 promoter. Addition of glucose to repress the promoter causes accumulation in the cytoplasm of proteins normally translocated into the

endoplasmic reticulum or mitochondria (Deshaies et al. 1988), so it appears that proteins of the hsp70 family are involved in this process. An activity purified from yeast, containing two 70kD proteins that comigrate with the products of SSA1 and SSA2, stimulates uptake of prepro- α -factor into microsomes in vitro (Chiriac et al. 1988). Together, these results suggest that hsp70 proteins might be the ATP-dependent 'unfoldase' required to keep translocated proteins in a unfolded conformation before they reach the membrane (Pelham, 1988).

Induction of the response: The heat shock response can also be induced by other stresses (reviewed by Lanks, 1986), including ethanol, arsenite, glucose deprivation and amino acid analogues. Like heat shock many of these inducers cause the denaturation of existing proteins or synthesis of defective proteins. For example, in Drosophila, mutations in actin genes induce hsp synthesis in flight muscle (Hiromi and Hotta, 1985). This is interesting in view of the proposed role of hsp70 and the observation that ubiquitin is heat inducible (Bond and Schlesinger, 1985).

Aberrant proteins in eukaryotic cells become attached to ubiquitin via their N termini, and become substrates for proteolysis. After heat shock, arsenite or amino acid analogue treatment, denatured protein overloads the system (Munro and Pelham, 1984 and 1985), leading to a high level of non-ubiquitinated denatured proteins - which might act as a trigger for the heat shock response.

Regulation of the response: The heat shock response of Drosophila is still the best characterised, and is regarded as the 'classical' response (see Ashburner and Bonner, 1979). Changes seen in the pattern of puffs in polytene chromosomes reflect an induction of hsp gene transcription within minutes of an increase in temperature (for example, from 25°C to 37°C), and a great reduction in transcription of 'normal' genes. Translation of normal mRNAs is also greatly reduced (although they are not degraded and remain in the cytoplasm), but the newly synthesised hsp mRNAs escape this general inhibition and are efficiently translated in the absence of competition from other mRNA species (Ashburner and Bonner, 1979). These two levels of regulation combine to ensure that the heat shock proteins are detectable within ten minutes of a temperature increase and rapidly become the major products of new protein synthesis.

The basic pattern of the heat shock response is similar in other organisms, but there are important differences. The most obvious is that the induction temperatures are related to the normal temperature of the organism. For example, the maximum response occurs between 36 and 37°C in Drosophila (Lindquist, 1980a), between 45 and 50°C in E. coli (Neidhardt et al. 1984) and at 65°C in Halobacteria (Daniels et al. 1982).

Transcriptional regulation: Studies of the promoters of heat shock genes have provided much of what is known about the organization of eukaryotic promoters in general. A high degree of conservation is demonstrated in the transcriptional regulation, as well as in the function of hsps. Transcription

of Drosophila heat shock genes is induced at the appropriate temperature for the host cells in: mammalian cells (Pelham, 1982); Xenopus oocytes (Voellmy and Rungger, 1982; Bienz and Pelham, 1982); sea urchin oocytes (McMahon^{et al.}, 1984); and even in tobacco (Spena et al., 1985).

Deletion studies by Pelham (1982) identified a conserved inverted repeat at positions -66 to -47. All heat shock genes - including hsp82 (Blackman and Meselson, 1986) and the small heat shock genes (Klemenz and Gehring, 1986) - contain at least one copy of this heat shock element (HSE). A single copy is sufficient to confer heat inducibility on the herpes simplex virus thymidine kinase gene in COS cells (Pelham and Bienz, 1982), although expression of Drosophila hsp70 genes requires at least the two proximal HSEs in some systems, including mouse Ltk- cells (Corces and Pellicer, 1984). The heat shock transcription factor has been identified and binds cooperatively to these sites (Parker and Topol, 1984). Interestingly, the distance of the HSEs from the transcription start site can be increased without affecting transcriptional efficiency (Biernz and Pelham, 1986), and the HSEs can also function bidirectionally (Amin et al., 1987; Key et al., 1986); in other words, they behave like enhancers.

Heat shock promoters also contain other elements, to each of which a specific protein binds. As well as HSEs and the obligatory TATA box, the Xenopus hsp70 gene (Biernz, 1986), for example, also has two CCAAT boxes - promoter elements found in globin genes and in the herpes tk gene, to which a multi-subunit protein binds (Chodos et al., 1988). A protein of 80-90kD binds to a 23bp hyphenated dyad in the region -600 to -450 of small heat shock genes which is required for

ecdysone induction (Cohen and Meselson, 1985; Riddihough and Pelham, 1986 and 1987). Similarly, different sequence elements, each of which interacts with a protein factor, are necessary for induction of human hsp70 transcription by high temperature and serum (B. Wu et al. 1986 and 1987).

I.B.ii Translational control of the heat shock response

Like other aspects of the heat shock response, the translational regulation is best characterized in Drosophila cells (see Ballinger and Pardue, 1985 for review). Similar preferential translation of hsp mRNAs compared with normal mRNAs has, however, been observed in other organisms, including Neurospora (Plesofsky-Vig and Brambl, 1985), Dictyostelium (Loomis and Wheeler, 1980), sea urchin embryos (Roccheri et al. 1981), and HeLa cells (Duncan and Hershey, 1984).

Within ten minutes of heat shock (at 37°C, the number of polysomes decreases dramatically - indicating that there is inhibition of translational initiation. The number of polysomes then increases as newly synthesized heat shock messages begin to be translated (McKenzie et al. 1975; Lindquist, 1980b). The rates of initiation and elongation on these heat shock mRNAs are similar to those on normal mRNAs in other systems that operate at 37°C, and are about five-fold higher than those in non-heat-shocked Drosophila cells. They correspond well with the values obtained by Hunt et al. (1989), who measured the effects of temperature on synthesis of globin in rabbit reticulocyte lysates (see Ballinger and Pardue, 1983 for discussion).

Translation of 'normal' mRNAs does not cease completely on heat shock, but the number of polysomes associated with messages encoding, for example, α - and β -tubulin and actin falls by 50-70%. Furthermore, the rate of elongation on these polysomes is reduced to 15-30-fold less than the polysomes translating heat shock messages. The result is that heat shocked cells contain a population of polysomes efficiently translating heat shock mRNAs and a second population very slowly translating normal mRNAs (Ballinger and Pardue, 1983). Translation of a final class of mRNA does appear to be blocked specifically and completely at the level of initiation: these mRNAs (including that encoding the ribosomal protein rp49) are found predominantly in the RNP fraction after heat shock.

These changes in the pattern of translation do not require either the transcription or the translation of hsp mRNAs: cells preincubated in actinomycin D stop translating normal mRNAs even though hsp mRNAs (and hence the proteins themselves) are not made (see Ballinger and Pardue, 1985).

Modifications to the translational apparatus: Translation in reticulocyte lysate demonstrates that (with the obvious addition of the heat shock proteins themselves) the mRNA in heat shocked cells directs the translation of the same set of proteins in the same amounts as mRNA from cells incubated at normal temperatures (Krüger and Benecke, 1981). The 25°C mRNA is therefore not degraded or modified to make it untranslatable during heat shock, and it is translated again after heat shock (see DiDomenico et al. 1982a). *Drosophila* cell-free systems have allowed some insight into the control of translation in vivo: lysates of cells grown at 25°C

translate both heat shock and normal mRNA efficiently; however, lysates of heat shocked cells translate heat shock mRNA preferentially (Storti et al. 1980; Krüger and Benecke, 1981). Because normal ribosomes are capable of translating heat shock messages there cannot simply be a 'switch' controlling translation of one or the other of two distinct populations of mRNA.

Translation of normal mRNA in lysates of heat shocked cells is rescued by addition of the crude ribosome fraction of 25°C cells, whereas addition of neither the ribosomal fraction nor the soluble fraction of heat shocked cells has any effect on translation in 25°C lysates (Scott and Pardue, 1981). This suggests that some component of the ribosomes (or a ribosome-associated factor) of heat shocked cells is modified in some way to inhibit translation of normal mRNAs. It also implies that the properties of the mRNAs that determine selection by ribosomes are resistant to protease digestion and phenol/chloroform extraction, and that they do not require the high temperature to be maintained (translations in both types of lysate were carried out at 28°C).

Unfortunately, these promising early experiments have not led to the isolation of the factor(s) modified by heat shock. Several changes have been reported to occur in the translational machinery on heat shock. In *Drosophila*, heat shock induces a rapid dephosphorylation of a ribosomal protein (Glover, 1982), which is apparently S6 - the protein whose phosphorylation state has been implicated in other examples of translational control (see I.A.iii). However, treatment with sodium arsenite, which induces hsp synthesis and inhibits translation of normal mRNAs, does not affect the

phosphorylation state of S6 (Olsen et al. 1983). In addition, detailed time-course experiments on HeLa cells have revealed that the inhibition of translation occurs before S6 is dephosphorylated (Tas and Martini, 1987). It seems therefore, that - as is the case on fertilization/maturation - there is no direct causal relationship between the phosphorylation state of S6 and the rate of translation.

In HeLa cells the inhibition of translation appears to be principally at the level of initiation, since polysomes disaggregate on heat shock. Using a fractionated in-vitro translation system, Duncan and Hershey (1984) have demonstrated that heat shock causes a reduction in the activity of eukaryotic initiation factors eIF-2, eIF-3, eIF-4F and eIF-4B. The factors eIF-2 α and eIF-2 β become phosphorylated, whereas eIF-4 becomes dephosphorylated. Unlike the situation in Drosophila, heat shocked HeLa cell lysates almost abolish translation when mixed with non-heat shocked lysates, indicating that they contain a dominant inhibitory factor - probably eIF-modifying enzymes.

Experiments on other mammalian systems provide more support for the involvement of initiation factors. In Ehrlich cells, eIF-2 α also becomes phosphorylated on heat shock; its phosphorylation state and the rate of protein synthesis return to normal levels with the same kinetics (Scorsone et al. 1987). Interestingly, synthesis of eIF-2 α itself is increased by about 40% by heat shock in rat thymocytes, indicating that the presence of functional eIF-2 α is needed to restore normal rates of protein synthesis (Colbert et al. 1987). The 90kD subunit of haem-regulated eIF-2 α kinase in Drosophila has sequence homology with hsp83 (Rose et al. 1987).

Importance of translational inhibition: The importance of translational inhibition varies between species, however. In some cell types, including Xenopus somatic cells, there is no dramatic reduction in overall protein synthesis (Bienz, 1982). There are conflicting reports about the role of translational control in HeLa cells. Although the studies discussed above suggest that, as in Drosophila, the rate of protein synthesis in HeLa cells falls immediately on heat shock (Hickey and Weber, 1982; Duncan and Hershey, 1984), other laboratories have reported no inhibition of overall protein synthesis at temperatures up to 45°C, above which translation of both normal and heat shock messages was inhibited (see for example Slater et al., 1981). It appears that the response may be related to the growth conditions and state of cellular differentiation.

A recent study by Jackson (1986) suggests that, far from being the typical case, the immediate repression of translation on heat shock observed in some Drosophila cell lines is not an integral and necessary part of the response. He found that in the Drosophila melanogaster KC 181 cell line, although the translation of a few mRNAs was specifically inhibited, translation of most normal mRNAs was not reduced on transfer to 37°C, but declined gradually over a three-hour period, as hsp synthesis increased. This suggests that the preferential translation of hsp70 could be the simple result of competition, and that it has higher affinity for the translational machinery.

Structure of heat shock mRNA: Whether or not there is a general inhibition of translation, there must be some features of heat shock mRNAs that allow them to be preferentially translated at high temperatures. Their 5' untranslated leader sequences are unusually long (160-250 bases), rich in A residues, and they have regions of homology at their extreme 5' ends and in the middle (see Lindquist and McGarry, 1986). If the *Drosophila* hsp70 mRNA leader is deleted, the RNA is translated at 25°C but not during heat shock (McGarry and Lindquist, 1985). If the first 95 residues are transferred to the 5' end of the alcohol dehydrogenase gene, the resulting RNA is translated during heat shock (Klemenz et al. 1985). However, deletions in either of the conserved elements have little effect on translation, whereas addition of 39 extra bases to the 5' end abolishes translation during heat shock (McGarry and Lindquist, 1985).

Lindquist and McGarry (1986) have suggested that the three dimensional structure of the leader, rather than its sequence, is important. However, even this hypothesis is difficult to reconcile with the observation that although 85% of the *Drosophila* hsp22 leader can be deleted without affecting translation, deletion of only the first 26 bases abolishes heat shock translation (Hultmark et al. 1986).

Termination of the response: The response is self-regulating. On return to normal temperatures, transcription of heat shock genes is inhibited and hsp mRNA is destabilized. Translation of normal mRNAs gradually resumes at the same time (DiDomenico et al. 1982a and b). However, this recovery occurs only when a certain amount of functional hsp (related to the length and

severity of the preceding heat shock) has been synthesised. If incubated in medium containing canavanine instead of arginine, cells produce non-functional hsp70, and both the inhibition of hsp70 synthesis and the resumption of normal protein synthesis are delayed. Deletion of the 3' end of hsp70 mRNA increases its stability during recovery (Simcox et al. 1985), indicating that 3'-end sequences are involved in the active destabilization of heat shock mRNA.

I.B.iii Different means to the same end

Lindquist (1985 and 1986) has argued that the differences in the relative importance of transcriptional and translational regulation are related to differences in cellular physiology. Because Drosophila is small and cold-blooded, changes in environmental temperature will immediately affect the temperature of the organism. Consequently, it must respond very rapidly - and does this immediately by changes in control of transcription and (in some species at least) translation.

Single-cell organisms must also respond rapidly. However, in Saccharomyces cerevisiae, for example, the half-life of mRNA is typically 10-40 minutes (compared with 6-9 hours in Drosophila), so competition can be reduced simply by allowing existing messages to decay in the absence of transcription. There is thus no need for specific inhibition of translation of normal mRNAs (Lindquist, 1981). The situation is similar in E. coli. Although heat shock proteins become the major translation products within 5-10 minutes of a shift to 50°C, this is again accomplished without the need for a block to general translation, since heat shock message is

detected within 15 seconds, and mRNA half lives are 1-5 minutes (Neidhardt et al. 1984).

Studies on translational control in larger organisms during heat shock have yielded confusing results. However, large animals are unlikely to encounter such rapid changes in body temperature. This is particularly true for warm-blooded organisms, where, even in fever, body temperature rises slowly and only by a few degrees, but it also applies to large cold-blooded animals. Consequently, it is probably not necessary to produce hsp's as rapidly as in other organisms. This may explain the less dramatic changes in translation observed in, for example, Xenopus (Bienz, 1982).

By contrast, the response in Xenopus oocytes appeared to be regulated entirely at the level of translation (Bienz and Gurdon, 1982). This would provide a very interesting example of adaption of the response to the needs of a particular cell type. However, recent results - including those presented in this thesis - have suggested that this is not so, and are discussed in detail in the following sections (I.B.v and I.C).

I.B.iv Developmental regulation of hsp expression

The heat shock response is developmentally regulated. This regulation has two aspects: in oocytes and early embryogenesis, heat shock proteins are not induced by high temperatures; however, at certain stages, they (or their cognates - it is often not clear which) are induced without heat shock. In general, acquisition of the ability to synthesize hsp's in response to heat shock occurs after fertilization, at different stages in different animals (see Heikkila et al. 1985a), and correlates well with the

acquisition of thermotolerance.

For example, the species of sea urchin Sphaerechinus granularis and Paracentrotus lividus can produce hsp's after hatching; Arbacia lixula can produce hsp's after the 64-128 cell stage. In all cases, ability to survive heat shock correlates with the ability to produce hsp's (Rocoheri et al. 1986). Induction of hsp's by mild heat shock also protects embryos from severe heat shock, but only for about three hours - even though the hsp's are present for longer than this (Soonso et al., 1986). In Strongylocentrotus purpuratus, however, one hsp is synthesized constitutively during embryogenesis: mRNA encoding hsp90 is present as 'masked' message in sea urchin eggs, and is translationally activated in non-heat-shocked embryos at the two-cell stage (Bédard and Brandhorst, 1986).

Mouse oocytes are very sensitive to heat shock, and synthesize no hsp70 (Curol et al. 1987), although they do make hsc70 and hsp89 - or proteins that comigrate with them. Proteins with identical mobility to hsp88 and hsp70 are made in two-cell embryos, and are probably the first products of embryonic transcription (Bensaude et al. 1983). Again, however, it is not clear whether these are hsp's or cognate proteins. It has recently been demonstrated that the 'hsp89' made in early embryos in fact consists of two proteins, one of which is constitutive and the other is heat inducible (Barnier et al. 1987). A 72kD cognate protein is also expressed at high levels in non-heat-shocked mouse embryo brains (Giebel et al. 1988).

Developmental regulation of heat shock gene expression is most clearly demonstrated in Drosophila and yeast (Kurtz et al. 1986). In Drosophila, messages encoding hsp26, hsp28 and hsp83 are transcribed in the ovarian nurse cells (at normal temperatures) and passed into the oocyte. Messages for hsp70, hsp68, hsp22 and hsp23 are not transcribed or induced by heat shock before the blastoderm stage, and embryos are sensitive to heat shock before this stage. Cognate proteins are produced in early embryos, and the small hsps themselves are induced later in embryogenesis by ecdysone (Zimmerman et al. 1983; and see I.B.1). In yeast, the situation is similar at sporulation: hsp26 and hsp64 are strongly expressed at normal temperatures and neither of the two hsp70s tested (SSA1 and SSA4) is induced by heat shock (Kurtz et al. 1986). The conservation of this regulation suggests that it may be a universal feature of gametogenesis. [Xenopus oocytes appeared to provide an exception to this pattern, but will be discussed in detail later (I.B.v).]

I.B.v The heat shock response in Xenopus

The heat shock response of Xenopus laevis somatic cells is similar to the 'classical' response of Drosophila in that the transcription of two major heat shock genes, encoding proteins of 30kD and 70kD, is dramatically induced by elevated temperatures. During a four-hour heat shock, these messages become the most abundant in the cell, and their translation products are prominent in the proteins synthesized at this time (Biens, 1982). However, unlike the case in Drosophila, the rate of translation of normal cellular messages is not greatly reduced by heat shock.

As in other species, synthesis of hsp's is very limited during early development, although the rate of normal protein synthesis is greatly reduced by heat shock. Bienz (1984a) reported that the hsp70 mRNA first becomes inducible at blastula (stage 9), whereas hsp30 mRNA is not induced detectably earlier than swimming tadpole (stage 42). Moreover, while different adult tissues produce constant amounts of hsp70 mRNA, induction of hsp30 mRNA varies considerably - kidney and gut cells produce about ten times as many transcripts per microgram of total RNA as do other tissues.

The acquisition of thermotolerance in embryos is correlated with the acquisition of the ability to synthesise hsp70: incubation at 35°C for 20 minutes before stage 8 invariably kills embryos, whereas 62% of blastulae and more than 90% of gastrulae survive this treatment (Haikkila *et al.* 1985b). Interestingly, the defects caused in partially thermotolerant embryos are confined to the vegetal hemisphere. Other hsp's have been observed in some, if not all, batches of embryos (Nickells and Browder, 1985). Partially thermotolerant embryos may also synthesise hsp83, and completely thermotolerant embryos produce hsp57 and hsp43. The latter two proteins are produced in greater amounts in the vegetal hemisphere, which also produces hsp35 - a unique hsp which is not produced in the animal half. It appears that the vegetal half requires these additional hsp's for complete thermotolerance.

The response in oocytes: Although the response of adult somatic cells and developing embryos does differ from the response in *Drosophila*, it has many features in common - in particular, the importance of transcriptional regulation. By contrast, exposing oocytes to elevated temperatures provided evidence that the response is regulated entirely at the level of translation (Bienz and Gurdon, 1982). For most of their experiments, Bienz and Gurdon used oocytes manually separated from *Xenopus* ovary. When these were incubated at temperatures above 25°C, the overall level of protein synthesis fell (unlike the situation in somatic cells; Bienz, 1982). Induction of hsp70 occurred at temperatures above 31°C, and was maximal between 33° and 35°C, and was detected by one- and two-dimensional SDS-polyacrylamide gel electrophoresis of oocytes incubated in [³⁵S]methionine. If oocytes were enucleated or incubated in α -amanitin (which inhibits transcription of RNA polymerase II) hsp70 could still be induced, indicating that its synthesis is not dependent on de novo transcription of oocyte hsp70 genes.

These results led to the suggestion that mRNA encoding hsp70 is stored in an untranslatable form, and is 'unmasked' on heat shock. As hsp70 synthesis was repeatedly induced when oocytes were heat shocked on five successive days, it appeared that the process was reversible, and that (unlike the situation in *Drosophila*) the hsp70 mRNA was not degraded during recovery. A further difference was that the recovery of normal protein synthesis occurred before hsp70 synthesis declined, implying that the response was not self-regulated in the same way as that in *Drosophila*.

When RNA extracted from heat shocked polysomes was translated in reticulocyte lysate, hsp70 was the major product detected on a two-dimensional gel. However, it was not possible to demonstrate conclusively that this mRNA was present before heat shock as a spot corresponding to hsp70 was barely discernible when total RNA was extracted from either heat shocked or control oocytes.

By measuring the amount of radioactive methionine incorporated, Bienz and Gurdon estimated that 10 fmol hsp70 was synthesized per heat shocked oocyte per hour. This would require between 10^7 and 10^8 copies of the mRNA per oocyte, which, they calculated, could not be transcribed in less than 10-100 days - even if transcription occurred at the maximum rate, and there are ten heat shock genes per haploid genome. In other words transcription of hsp70 genes during heat shock cannot possibly account for the observed synthesis of hsp70. The translational control in oocytes therefore appeared to be another example of adaptation of the response to the needs of a particular cell type - in this case one with an extremely high ratio of cytoplasm to nucleus.

Recent experiments have cast serious doubts on these conclusions, however. King and Davis (1987) have suggested that all the hsp70 detected when oocytes are heat shocked is in fact produced by the follicle cells that invariably remain attached throughout the incubation and analysis. They found that defolliculated oocytes did not synthesize detectable amounts of hsp70 at any temperature, whereas heat shock did induce hsp70 synthesis in the follicle cells that had been removed.

Biens and Gurdon (1982) had also investigated the effect of defolliculation but found that, although it reduced the synthesis of hsp70, it did not abolish it. As actin synthesis was reduced by the same proportion, they concluded that the effect was due to a general reduction in viability of defolliculated oocytes. The results of King and Davis would however suggest that the hsp70 detected had in fact been synthesized in follicle cells remaining attached to the oocytes. They used a method of defolliculation that involved incubation in collagenase followed by manual removal of the inner cell layer in a hypertonic buffer, and demonstrated by histological examination that all cells had been removed. In contrast, when they used the one-step defolliculation method of Biens and Gurdon, up to 30% of the cells remained.

King and Davis failed to detect hsp70 in the in vitro translation products of polysomal mRNA extracted from defolliculated oocytes, and suggest that, like oocytes of other species, they neither synthesize nor require heat shock proteins. Biens (1984a) did detect sequences complementary to hsp70 DNA in oocytes by S₁ assay. It was not clear however, whether these encoded hsp70 itself, or heat shock cognate protein(s). (Proteins of electrophoretic mobility very similar to hsp70 which were expressed at normal temperatures were detected in oocytes by both Biens and Gurdon, and King and Davis.)

The picture has been further complicated by reports from other groups apparently describing a completely different response. Browder et al (1987) find that heat shock induces synthesis of hsps of 83, 78, 70 and 57 kD in oocytes. Removal of follicle cells by treatment with collagenase in medium

supplemented with pyruvate and oxaloacetate does not affect their synthesis, and naturally defolliculated body cavity eggs also undergo the response (and produce two additional hsps of 22 and 16 kD). Fertilization terminates the heat shock response, and hsps are not induced in embryos before the blastula stage. Browder et al find that this response is dependent on incubation in supplemented medium; in unsupplemented medium oocytes consistently respond in the manner described by Bienz and Gurdon and by King and Davis. This type of response has also been observed by Guedon et al (1985), who investigated the possible role of diadenosine tri- and tetraphosphates in the induction of the response. They found that injection of diadenosine tetraphosphate after a mild heat shock specifically enhances the synthesis of hsp70, and its injection before heat shock inhibits induction of all hsps except hsp70, suggesting that it has a role in regulation of the response.

These conflicting results leave many issues unresolved. It is not clear what is the 'normal' pattern of protein synthesis during heat shock in Xenopus oocytes. It is not known what is the relative contribution of oocytes and follicle cells to the pattern observed. And it is not known at what level the response in oocytes (if any) is regulated.

I.C THE WORK IN THIS THESIS

I.C.1 Aim and strategy

The original aim of this project arose from the experiments of Bienz and Gurdon (1982), which suggested that the heat shock response of Xenopus oocytes is controlled entirely at the level of translation: hsp70 mRNA appeared to be masked at normal temperatures and preferentially translated during heat shock. It therefore seemed that the heat shock response of oocytes would provide a useful model system for studying translational regulation in development, in the same way as the response in other cell types had yielded much information about the control of transcription. In particular, it was hoped that the ability of oocytes to unmask and re-mask mRNA reversibly might allow the design of experiments to investigate the kinds of mechanism involved in the repression of protein synthesis during oogenesis and the stimulation of maternal mRNA translation that occurs on maturation and fertilization.

The initial aim was therefore to identify the sequences involved in masking of hsp70 mRNA in oocytes at normal temperatures and/or those allowing preferential translation during heat shock. This was to be done by exploiting the well characterized capacity of Xenopus oocytes to act as a transcription/translation system.

Many protein-coding genes injected into oocyte nuclei are transcribed by the maternal store of RNA polymerase II. Transcripts initiated and terminated at the correct places are exported to the cytoplasm where they are translated and the resulting polypeptides are correctly modified by, for example, removal of signal peptide, glycosylation or phosphorylation

(Colman 1984a and b). Although some aberrant transcripts are also synthesized, these are generally degraded in the nucleus. However, some injected genes are not transcribed detectably in oocytes, presumably because necessary transcription factors are not present. In addition, injection of genes that do not contain introns (such as hsp70) has been more successful than for intron-containing genes, perhaps because incorrect splicing occurs. Many exogenous mRNAs are also translated well when injected directly into oocyte cytoplasm (Colman, 1984b).

Injection of DNA or RNA into oocytes has traditionally been used as a method of producing large amounts of protein, to study mechanisms such as secretion, rather than as a system in which to study the control of transcription or translation. However, there are examples of injected genes that have been regulated appropriately: for example, Drosophila hsp70 genes are transcribed only during heat shock (Bienz and Pelham, 1982), whereas Xenopus hsp70 genes are transcribed constitutively (Bienz, 1984b). A recent study has found that synthetic, polyadenylated RNA injected into oocytes is deadenylated and released from polysomes on maturation, in the same way as the endogenous mRNA (Hyman and Wormington, 1988).

Bienz (1984b) demonstrated that Xenopus hsp70 genes are efficiently transcribed if injected into oocytes both at normal temperatures and during heat shock. It was therefore intended to modify the region of these genes corresponding to the 5' untranslated region of mRNA, inject the modified genes into oocytes and follow translation of the resulting RNA at normal and heat shock temperatures. As an alternative approach to DNA injection, modified hsp70 RNAs were to be synthesized in vitro and injected into oocytes. This would have the

advantage that the quantity of RNA introduced could be controlled directly. For both approaches, it was desirable to alter the coding region so that the gene product could be distinguished from the endogenous hsp70. Two constructs were planned: firstly, one coding for a truncated protein; and secondly, one coding for a fusion of the N-terminal end of hsp70 with chimpanzee α -globin.

I.C.ii The work in this thesis

Before embarking on the strategy outlined above, the heat shock response of oocytes and their attached follicle cells was re-examined. This became particularly necessary in the light of the results of King and Davis (1987), which suggested that oocytes were incapable of synthesizing hsp70, even during heat shock. A critical difference between their results and earlier results was that, while Biers and Gurdon (1982) found that defolliculated oocytes did synthesize hsp70 during heat shock, King and Davis found that they did not. It seemed possible that some methods of defolliculation might not remove all follicle cells. Chapter III describes a new procedure devised to remove reliably all these cells, and its use in experiments to analyse protein synthesis in oocytes both with and without follicle cells, at normal temperatures and during heat shock.

Two-dimensional gels of oocytes labelled with [³⁵S]methionine revealed that at least two prominent proteins of mobility very similar to hsp70 (possibly heat shock cognates) are made in all batches of oocytes at normal temperature. Their rate of synthesis is reduced by heat shock, usually by the same proportion as the (previously

demonstrated) dramatic reduction in the overall rate of protein synthesis. However, while hsp70 is the major protein produced during heat shock when follicle cells are present, it is not detectable when they are removed, whether this is done before or after the labelling period. All the hsp70 detected is therefore made in the follicle cells. If it is synthesized in oocytes, it is at levels too low to detect by this method.

In some experiments, the alternative pattern of protein synthesis occurred (see Browder et al. 1987), in which the overall rate of protein synthesis does not fall and synthesis of certain proteins is induced in oocytes. However, none of these correspond with bona fide hsp70, although two do have mobility very similar to hsp70.

An RNase protection assay of oocytes with and without follicle cells demonstrated that, as expected, transcription of hsp70 mRNA is dramatically induced in follicle cells. By contrast, each oocyte contains a small amount of hsp70 mRNA (1000-fold less than is induced in its follicle cells) which is not increased by heat shock.

To investigate whether oocytes are capable of translating hsp70 mRNA, DNA encoding hsp70A, hsp70B, and a fusion of hsp70B with globin were injected (Chapter IV). These were transcribed efficiently and yielded RNA of the predicted sizes - identical (in the cases of the native genes) to the transcripts induced in follicle cells. The proportion of transcripts with poly(A) tails long enough to bind oligo-dT cellulose was also similar to follicle cell hsp70 mRNA.

Surprisingly, transcription of all the injected genes was induced by heat shock, the effect being most dramatic for hsp70B, which was transcribed much less efficiently than

hsp70A at 20°C. However, the transcripts induced on heat shock remained in the nucleus, so that the amount available for translation was the same at both temperatures.

Results of labelling protein synthesized by injected oocytes are reported in Chapter V. Unfortunately, the fusion protein was only detected in one experiment, possibly because, since it was derived from hsp70B, relatively few cytoplasmically located transcripts were available for translation. Synthesis of hsp70A was detected in defolliculated oocytes incubated at 20°C. In heat shocked oocytes, the rate of translation of hsp70 mRNA was usually (but not always) at a slightly lower rate than at normal temperatures. However, it was always preferentially translated compared with other mRNA species. Occasionally, the hsp70 protein self-degraded during analysis. Oocytes undergoing the unusual heat response also synthesized hsp70 when hsp70A genes were injected, although it was not clear whether it was preferentially translated during heat shock since the overall rate of protein synthesis did not fall.

These results suggested that oocytes can treat hsp70 mRNA 'physiologically', and that they might be used to attempt to identify translational control signals. Injection of RNA into the cytoplasm provides a means of controlling directly the amount of RNA available for translation under all conditions of the experiment. In a series of experiments to test the feasibility of this approach to study hsp70 synthesis, hsp70 RNA was transcribed *in vitro* and injected into oocytes (Chapter VI). However, although the plasmids used as templates for transcription were modified so that the RNA transcribed was as near as possible in structure to native

hsp70 mRNA, it was not translated preferentially during heat shock. These results suggest that when these RNAs are injected into oocyte cytoplasm they are not subject to the same regulation of translation as RNA transcribed from DNA injected into the nucleus.

Chapter VII describes experiments that initially appeared to offer a short cut to identifying RNA sequences involved in preferential control during heat shock. When DNA encoding influenza nucleoprotein NP under control of the thymidine kinase (TK) promoter was injected into oocytes, the RNA transcribed was preferentially translated during heat shock by some batches of oocytes. Comparison of sequences revealed a region of almost complete homology in the 5' untranslated regions of TKNP RNA and hsp70 mRNA. A double-stranded oligonucleotide with this sequence has been synthesized and inserted into another gene (preprochymosin), also under the thymidine kinase promoter, to investigate whether it can act autonomously to confer preferential translation.

Natural flu NP mRNA also contains the consensus sequence, although it is nearer the 5' cap structure than in the other RNAs. However, it was never translated preferentially when injected into oocytes, again suggesting that the method of introducing these exogenous RNA into oocytes might be important in determining its translational regulation.

CHAPTER II: MATERIALS AND METHODS

II.A MATERIALS

II.A.i Sources of reagents

General chemicals: Most standard chemicals were AnalaR grade, or equivalent, and were obtained from BDH Chemicals Ltd, the Sigma Chemical Company Ltd, May and Baker Ltd, and Fisons Scientific plc. Nitrocellulose manufactured by Schleicher and Schüll was purchased from Anderman and Co; oligo-dT cellulose was from Collaborative Research. All nucleotides (dNTPs, ddNTPs, rNTPs, and the cap analogues) were obtained from Pharmacia.

Radioisotopes: These were all obtained from Amersham International plc, and were all in aqueous solution -

[α - 32 P]dGTP and dCTP, at a specific activity of 3000Ci/mmol, and 10mCi/ml;
[α - 35 S]dATP α S, at >1000Ci/mmol, and 10mCi/ml;
[γ - 32 P]ATP at 5000Ci/mmol, and 10mCi/ml;
[α - 32 P]UTP at 800Ci/mmol, and 20mCi/ml ;
L-[35 S]methionine at 1300Ci/mmol, and 15mCi/ml;
[14 C]-labelled protein molecular weight markers.

Enzymes: Restriction endonucleases were purchased from Amersham International plc, Bethesda Research Laboratories (UK), Boehringer-Mannheim, and New England Biolabs. T4 DNA ligase was obtained from T. Hunt (University of Cambridge); T4 polynucleotide kinase and *E. coli* DNA polymerase I (Kornberg enzyme) were from Amersham International plc; Klenow fragment of DNA polymerase I was from Pharmacia; CIP was from

Boehringer-Mannheim. SP6 RNA polymerase was purchased from
Boehringer-Mannheim and New England Nuclear; T7 RNA polymerase
and RNasin were from Pharmacia. RNase A and RNase T1 were from
Sigma. Lysozyme was purchased from Sigma; proteinase K was
from Boehringer-Mannheim.

Plasmids: The following plasmids were used -

phsp70A and phsp70B (kind gift of M. Bienz, Cambridge);
pTKNP, pTK82, pTK82*, pTKOv (kind gifts of J. Davey, R.
Strachan, and A. Colman, University of Warwick);
pGOV₂
(kind gift of V. Lingappa, UCSF);
pSP64T (kind gift of P. Krieg, University of Harvard);
pGEM-1 and pGEM-2 (Promega Biotech);
M13mp18 and M13mp19 (Pharmacia).

Oligonucleotides: Most oligonucleotides were synthesized in
the Department on an Applied Biosystems synthesizer, by Mrs G.
Soott. The 'translational stop' oligonucleotide and the M13
sequencing primer were both obtained from Pharmacia.

In vitro translation systems: Reticulocyte lysate was from T.
Hunt (University of Cambridge); Wheatgerm was a kind gift of
D. Lightfoot (University of Warwick).

Antibodies: The following antibodies were kind gifts -

rabbit anti-prothymosin (R. Strachan, University of
Warwick; Strachan, 1986);
rabbit anti-hen lysozyme (D. Cutler, University of Warwick;
Cutler, 1982);
rabbit anti-influenza (A. Carver, University of Warwick);
mouse anti-NP (5/1, I. Jones, University of Oxford).

Rabbit anti-human haemoglobin was purchased from Miles-Yeda Ltd, Israel.

Electrophoresis reagents: Tris for SDS-PAGE was BDH Aristar grade; glycine was also from BDH; bisacrylamide was purchased from Eastman-Kodak; agarose (type II) was from Sigma.

Autoradiography and photography: En³Hance was purchased from New England Nuclear; intensifying screens were from Dupont; X-ray film was Fuji RX. Autoradiographs were photographed using Kodak Panatomic X, which was developed with Ilford Microphen. Prints were made on Kodak paper, grades 2 and 3, using Kodak chemicals.

Frogs and oocytes: Frogs were purchased from the South African Snake Farm; MS222, Hoescht 33258 dye, collagenase (type IV), and sheep hyaluronidase were all from Sigma.

II.A.ii Preparation of buffers, media and standard reagents

5xTBE - 0.45M Tris, 0.45M boric acid, 10mM EDTA, pH8.3.
20xTAE - 0.8M Tris, 0.4M sodium acetate, 40mM EDTA, pH8.3.

10xMOPS - 0.2M MOPS, 0.05M sodium acetate, 0.01M EDTA, pH7.0.

TE - 10mM Tris-HCl, 1mM EDTA, ^{or} pH7/8.

20xSSC - 3M NaCl, 0.3M sodium acetate, pH7.0.

20xSSPE - 3M NaCl, 0.2M NaH₂PO₄, 20mM EDTA.

50xDenhardt's - 1% ficoll, 1% polyvinylpyrrolidone, 1% BSA.

L broth - 1% tryptone, 0.5% yeast extract, 0.5% NaCl.

2xTY - 1.6%(w/v) tryptone, 1% yeast extract, 0.5% NaCl.

II.B AGAROSE GEL ELECTROPHORESIS OF DNA

In sections II.B-D, I describe the general techniques used in the construction and large scale preparation of plasmids. The precise details of construction for each plasmid are given in the appropriate Results and Discussion chapter. DNA manipulation was carried out using standard protocols, most of which are described in detail by Maniatis *et al* (1982), although the exact conditions of the reactions were occasionally varied slightly.

II.B.1 Agarose gels

Fragments of DNA were analysed on horizontal agarose gels at various points during the subcloning procedure, and after restriction digest of the final product. The percentage of agarose included was varied according to the sizes of the fragments, but was usually between 1 and 2% [see Maniatis *et al* (1982) for the range of sizes of DNA molecules separated by different percentage gels]. Two different types of gel were used.

(1) In earlier experiments, the agarose was melted in 1xTAE buffer. This was poured onto a glass plate (approximately 140mm x 200mm), and wells of 10-20 μ l formed by inserting a comb before the gel set. Samples of DNA were prepared by adding 0.25 volume of TAE loading buffer (0.5xTAE, 25mM EDTA, 50% glycerol, 0.1% bromophenol blue). After the samples had been loaded, the gel was electrophoresed at 100-150V in 1xTAE, using wicks made of three thicknesses of 3MM paper. Electrophoresis was usually for 30-120 min, depending on the sizes of fragments to be resolved. The gel

was then stained with ethidium bromide at about $2\mu\text{g}/\text{ml}$ ⁱⁿ $1\times\text{TAE}$ for 15-20 min (destaining was not usually necessary), and DNA was visualized by placing the gel on a UV light box. 50-100ng in a single band was clearly visible. If a permanent record was required, the gel was photographed using a polaroid camera.

(2) In later experiments, a commercial minigel apparatus was used for DNA electrophoresis. The wells were narrower, so 10-20ng in a single band was easily visible, and the resolution was good enough for most purposes. The buffer used for these gels was $0.5\times\text{TBE}$ and ethidium bromide was added at $0.5\mu\text{g}/\text{ml}$ to the gel mix just before it was poured. Samples were prepared by adding 0.25 volumes TBE loading buffer ($5\times\text{TBE}$, 0.2% SDS, 50mM EDTA, 20% glycerol, 0.1% bromophenol blue), loaded, and the gel was electrophoresed, submerged in $0.5\times\text{TBE}$ containing $0.5\mu\text{g}/\text{ml}$ ethidium bromide, at 25-100V for 30-120 min. The DNA bands were then visualized and photographed as before.

Size markers: The choice of markers depended on the size range of the fragments to be resolved. Typically, pAT153 digested with HinfI (75-1631bp) or λ DNA digested with EcoRI and HindIII (0.125-21.23kbp) were used. Exact sizes of these markers are given by Old and Primrose (1985).

Amount markers: These were prepared by determining the concentration of a solution of linearized, RNA-free pGEM-1 by measuring its A_{260} . When the concentration of a small volume of DNA had to be estimated, a $1\mu\text{l}$ sample was run on an agarose gel, and with a range of these markers (typically from

25-250ng/track), and the fluorescences were compared.

II.B.ii Purification of DNA from agarose gels

The DNA was electrophoresed by either of the methods described above, then visualized on a UV light box. A slit was made in the gel just below the band of interest. Into this was inserted a narrow strip of Whatman no.1 paper, backed by a single thickness of dialysis membrane. A second strip of dialysis membrane was inserted into a slit cut above the band, to prevent contamination with any larger fragments. The gel was then run, with wicks, at 1kOV until the DNA had all run onto the paper. The paper and dialysis membrane were placed in a small Eppendorf tube, in which a hole had been pierced with a sterile needle. This was put into a large Eppendorf tube, without a cap, and centrifuged for 20s. The eluate was removed from the large tube, and the paper washed twice with 100 μ l of TE, being centrifuged for 20s each time.

Recovery of DNA: The DNA was recovered from the pooled eluates by extraction with phenol [prepared as described by Maniatis et al. (1982)] and chloroform followed by ethanol precipitation. These standard techniques are described in detail in Maniatis et al (1982). Briefly, an equal volume of phenol/chloroform(1:1) was added, vortexed, and the phases separated by centrifugation. The aqueous phase was then extracted again with diethyl ether to remove any traces of phenol. 0.1 volume of either 3M sodium acetate pH5.5 or 3M NaCl was then added, and the DNA was precipitated by adding 2-2.5 volumes ethanol, and storing the mix at -20°C for at least two hours, or at -70°C for 30-60 min. It was then

centrifuged in a microfuge for 10 min. The resulting pellet was then washed by adding ice-cold 80% ethanol, and the pellet was dried under vacuum before being taken up in water or the appropriate buffer.

This basic protocol was usually followed when DNA or RNA was extracted with phenol/chloroform or precipitated.

II.C. USE OF DNA MODIFICATION ENZYMES

Various modification enzymes were used during subcloning and analysis of DNA. Often several modifications were carried out sequentially, and the DNA was usually phenol/chloroform extracted and ethanol precipitated between each step. However, if the reaction conditions of the two enzymes were compatible, this was unnecessary.

II.C.i. Restriction enzyme digestion

In general, restriction digests were carried out using the low, medium, and high salt buffers described by Maniatis et al:

| Buffer | Tris-HCl pH7.5 | NaCl | MgCl ₂ | DTT |
|--------|----------------|-------|-------------------|-----|
| Low | 10mM | 0 | 10mM | 1mM |
| Medium | 10mM | 50mM | 10mM | 1mM |
| High | 50mM | 100mM | 10mM | 0 |

For enzymes (notably SmaI and XbaI) that do not work well in any of these buffers, buffers were made up according to the manufacturers' instructions.

The appropriate buffer was added as a 10x stock to DNA and water, so that the final concentration of DNA was about 100µg/ml. Often, BSA and spermidine were also included in the reaction, at final concentrations of 100µg/ml and 4mM

respectively, as they improved the efficiency of digestion. When miniprep DNA was analysed, RNase A was included at 100 μ g/ml. The restriction enzyme was then added (usually in 2-10-fold excess) and the mixture incubated at the appropriate temperature for at least 1h. Samples of digests in low and medium salt buffer were usually run directly on agarose gels, but high salt digests were first phenol/chloroform extracted and ethanol precipitated.

II.C.ii Treatment with Klenow

The Klenow fragment of DNA polymerase I was often used to fill in the sticky ends of DNA fragments left with 5' overhangs after restriction digest, prior to ligation with other blunt-ended fragments. Reactions were typically 50 μ l, and contained up to 2.5 μ g DNA in 1x medium salt buffer, 0.5mM each necessary dNTP. Occasionally a trace of [α - 32 P]dCTP or [α - 32 P]dGTP was also included, either to check that the Klenow was working or to allow small amounts of DNA to be analysed by gel electrophoresis. 2 units Klenow were then added and the mixture was incubated at room temperature for 30 min.

Synthesis of radioactive DNA markers: DNA markers made by digestion with an enzyme leaving 5' overhangs containing dCTP or dGTP could be labelled using Klenow, for use on sequencing-type gels (see II.E.iii). A 20 μ l reaction was set up including 50 μ g DNA (e.g. pBR322 digested with HpaII), 1x medium salt buffer, 30 μ Ci [α - 32 P]dGTP or [α - 32 P]dCTP, and 2.5mM each of all other necessary dNTPs. 2 units Klenow were added and the mix was incubated at room temperature for 30-45 min. The DNA was recovered by phenol/chloroform extraction and

ethanol precipitation.

II.C.iii Phosphatase treatment

Vector DNA, containing the ampicillin resistance marker, was treated with calf intestinal phosphatase (CIP) before ligation with insert, to prevent its religation. To 50 μ l DNA in H₂O or restriction buffer were added 6 μ l Tris-HCl pH8.0, 1 μ l 10% SDS, and 5 μ l (2.5 units) CIP. This was incubated at 37°C for at least 2h, after which it was extracted twice with phenol/chloroform, once with ether, and finally ethanol precipitated. This treatment was effective for at least 7 μ g DNA.

II.C.iv Ligation

DNA fragments were ligated in 10 μ l reactions which contained 50-100ng of each fragment, in 50mM Tris-HCl pH7.4, 10mM MgCl₂, 10mM DTT, 1mM spermidine, 1mM ATP. After addition of 2 units T4 DNA ligase, the mix was incubated at 15°C for 4-20 h. Control ligations were also set up, containing CIP-treated vector but no insert DNA. A small aliquot of each ligation was then transformed into E. coli.

II.D TRANSFORMATIONS AND PREPARATION OF PLASMID DNA

II.D.i Transformations

1ml of an overnight culture of E. coli DH1 cells was added to 50ml L broth (see II.A.ii), and incubated at 37°C in a shaking incubator until its A₅₄₀ was about 0.3. The cells were then left on ice for 30 min, pelleted by centrifugation, and resuspended in 25ml ice-cold 100mM MgCl₂ (freshly diluted from 1M stock solution). The cells were immediately centrifuged again, and the pellet was then taken up in 2.5ml

ice-cold 100mM CaCl₂ (freshly made up). The cells were then left on ice for 1-4 h before transformation.

Each DNA sample (typically 1 μ l of a ligation mix) was added to 100 μ l of Tris-HCl pH7.4, mixed with 200 μ l competent cells and incubated on ice. Control transformations contained no DNA or 500pg of untreated plasmid (to give an estimation of transformation efficiency). After 30 min on ice, the tubes were heat shocked for 2 min at 42°C, and returned to ice for a further 30 min. 0.5ml L broth was added to each tube, and they were incubated at 37°C for 30 min. As all the plasmids used in this study contained a gene conferring resistance to ampicillin, transformed bacteria were selected by spreading transformation mixes on dried L agar plates containing ampicillin at 50 μ g/ml. The plates were incubated overnight at 37°C.

II.D.ii Mini plasmid prep

2ml aliquots of L broth containing 100 μ g/ml ampicillin were inoculated with ampicillin-resistant colonies and the cultures grown overnight at 37°C. The bacteria were pelleted by centrifuging briefly in a minifuge, and plasmid DNA was prepared by a method based on the alkaline lysis method of Birnboim and Doly (1979)(see Maniatis *et al.* 1982). Each pellet was resuspended in 100 μ l of 50mM glucose, 25mM Tris-HCl pH8.0, 10mM EDTA, 2mg/ml lysozyme (added just before use). After 5 min at room temperature, 200 μ l of 0.2M NaOH, 1% SDS were added, and mixed without vortexing. After incubation on ice for 5 min, 150 μ l of 3M sodium acetate pH4.8 were added. The samples were vortexed gently then left on ice for a further 5 min before being centrifuged in a minifuge for 1

min. The supernatants (about 380 μ l) were transferred to new tubes, and the plasmid DNA was precipitated by adding 0.54 volume (195 μ l) of isopropanol, mixing and leaving the samples at room temperature for 2 min. They were centrifuged for 1 min, and the pellets were washed in 80% ethanol, dried and taken up in 20 μ l TE. The DNA was analysed by digesting an aliquot (typically 2 μ l) with appropriate restriction enzymes, including BSA, spermidine, and RNase A in the reaction (see II.C.1).

II.D.iii Large-scale plasmid preparation

An overnight culture of bacteria was used to inoculate 250-500ml L broth plus ampicillin (at 100 μ g/ml), and incubated with shaking at 37°C for about 4 h until the A_{600} was approximately 0.8. If the plasmid was present at high copy-number, the bacteria were pelleted immediately. However, for low copy-number plasmids (in this study, only the pBR322-based pTK constructs) an amplification step was required: chloramphenicol was added to 100 μ g/ml and the incubation was continued overnight. Plasmid DNA was then prepared by one of the two following methods.

Alkaline lysis: This is an adaptation by Paul Krieg of the method of Birnboim and Doly (1979). The bacterial pellet was resuspended in 4ml of 25mM Tris-HCl pH8.0, 10mM EDTA, 15% sucrose, 2mg/ml lysozyme. This was transferred to a polypropylene Oakridge tube and incubated on ice for 20-40 min. Next, were added 8ml 0.2M NaOH, 1% SDS. After a further 10 min on ice, 5ml 3M sodium acetate pH4.6 were added, and the mixture was left on ice for a further 40 min. It was then

centrifuged at 18000rpm in the 8x50 rotor of an MSE HS18 centrifuge at 4°C for 30 min. The supernatant was transferred to a fresh tube; 100µg RNase A was added and it was incubated at 37°C for 30 min. After one phenol/chloroform extraction and one chloroform extraction, the plasmid DNA was ethanol precipitated. The resulting pellet was taken up in 5ml H₂O, made 2.5M in ammonium acetate, and ethanol precipitated again. It was finally taken up in 0.5ml H₂O. At this stage, the DNA could be injected into oocytes. However, it was usually further purified by centrifugation in caesium chloride gradients (see II.D.iv).

Triton lysis method: This procedure was based on the method of Clewell and Helmsinki (1989). The bacterial pellet was taken up in 12ml 25% sucrose, 50mM Tris-HCl pH8.0. To this were added 2.5ml of a fresh 10mg/ml solution of lysosyme. After a 5 min incubation on ice, 2.5ml of 0.5M EDTA were added and the suspension incubated on ice for a further 5 min. Next were added 20ml triton lysis solution (50mM Tris-HCl pH8.0, 62.5mM EDTA, 0.1% Triton X-100). The lysate was then centrifuged for 30 min in 50ml polypropylene tubes at 18000rpm and 4°C in the 8x50 rotor of an MSE HS18 centrifuge. The supernatant (cleared lysate) was extracted twice with phenol/chloroform, and once with chloroform, then dialysed for 2 h in 10 l of TE, with one change of medium. It was made 0.3M in NaCl and ethanol precipitated overnight. The plasmid pellet was then taken up in 10ml H₂O, made 0.3M in NaCl and the RNA at least partially removed by adding 50µl of RNase A at 10mg/ml, and incubating at 37°C for 30 min. Finally, the RNase was removed by phenol/chloroform extraction, and the solution was extracted

once with chloroform then ethanol precipitated. The DNA was recovered by centrifugation as usual, and taken up in 5ml 0.1xSSC. Before microinjection into oocytes, it was purified further by CsCl density gradient centrifugation.

II.D.iv Cesium chloride density gradients

For each plasmid, 26g CsCl was dissolved in 20ml 0.1xSSC. 25ml of this was added to 5ml DNA and 1.5ml 10mg/ml ethidium bromide in a 50ml Quickseal tube. The tubes were balanced with remaining CsCl solution, filled with liquid paraffin and sealed. They were centrifuged overnight at 45000rpm at 23°C in the VT.50 rotor in a Beckman L8 centrifuge. The nucleic acid was visualized in UV light and the plasmid band recovered using a syringe. Ethidium bromide was removed by five extractions with isoamylalcohol. The aqueous phase was dialysed for at least 2 h in 10 l TE, with one change of medium, then made 0.3M in NaCl, and ethanol precipitated.

The repeated phenol/chloroform extractions described above were occasionally avoided by loading the cleared lysate directly onto CsCl gradients: 1g CsCl was added per ml of lysate, and the gradients were set up and run as before. As this first gradient contained so much RNA, the plasmid band was recovered and transferred to a 12ml tube. A second gradient was run overnight in a 70.1T. rotor in a Beckman L8, at 40000rpm. The plasmid band was then removed, extracted, dialysed and precipitated as before.

II.E DNA SEQUENCING

II.E.1 Sequencing single-stranded M13 DNA

This was carried out using the methods described in the Amersham DNA sequencing handbook, as modified by John Shuttleworth.

Insertion of DNA into M13: The DNA fragment of interest was inserted into the double-stranded replicative form of the vectors M13mp18 and mp19 (which contain the multiple cloning site in opposite orientations). The appropriate vector was linearized with appropriate restriction enzyme(s), compatible with the ends of the fragment to be inserted. Ligations were set up as described (II.C.iv), using 50ng vector DNA, although this was not phosphatase treated.

Transformation: An overnight culture of *E. coli* TG2 cells in 2XTY (II.A.ii) was set up from a single colony. 40ml 2xTY was inoculated with 2ml of this culture, and shaken at 37°C for about 2 h, until its A₅₅₀ was 0.3. The cells were gently centrifuged and the pellet taken up in 20ml fresh, ice-cold 50mM CaCl₂. This was left on ice for 20 min, then the cells were pelleted again, and resuspended in 4ml ice-cold 50mM CaCl₂.

Aliquots of 0.3ml of competent cells were transferred to 15ml sterile culture tubes. 5µl ligation mix were added to each. Control transformations were: 25ng cut vector; 25ng cut and religated vector; and 1ng uncut vector. All transformations were incubated on ice for at least 40 min, heat shocked at 42°C for 3 min, and returned to ice.

A mixture of the following was prepared for each tube: 40 μ l 100mM IPTG; 40 μ l 2% X-gal in DMF; 200 μ l 2xTY,* 270 μ l of this was added to each tube of cells. They were then plated out by adding to each 3ml molten H top agar [1%(w/v) tryptone, 0.8% NaCl, 0.8% agar], mixing, and pouring onto a prewarmed H agar plate [1%(w/v) tryptone, 0.8% NaCl, 1.2% agar]. The plates were incubated at 37°C overnight.

Preparation of single-stranded DNA: 100ml 2xTY was inoculated with 1ml of an overnight culture of TG2 cells. White plaques (in which the phage β -galactosidase gene had been inactivated by the insertion of the DNA of interest) were picked into 1.5ml aliquots of the diluted culture. These were shaken at 37°C for 5 h. The bacteria were pelleted by centrifugation in a minifuge, and the supernatants were decanted into fresh tubes, recentrifuged and decanted again, then 100 μ l was removed and stored at -20°C. To the remainder was added 200 μ l of 20% polyethylene glycol 6000, 2.5M NaCl. The tubes were vortexed, left to stand at room temperature for 15 min, then centrifuged for 5 min. The supernatants were discarded and the tubes centrifuged for a further 2 min. Any remaining liquid was carefully removed, and the viral pellets taken up in 100 μ l TE and 50 μ l phenol. The tubes were vortexed, left at room temperature for 15 min, vortexed again, then centrifuged for 3 min. The aqueous phases were transferred to fresh tubes and the DNA was precipitated by the addition of 10 μ l 3M sodium acetate and 250 μ l ethanol. After overnight incubation at -20°C, the DNA was recovered by centrifugation as usual and finally taken up in 40 μ l TE.

*It was not necessary to add more bacteria at this stage as sufficient were already present to form a lawn.

Before sequencing, 5 μ l of each single-stranded preparation was run on a 1% agarose gel, along with a sample of single-stranded vector, to check that they did contain inserts.

Sequencing reaction: This was carried out by the method described by Sanger et al (1977). The template was annealed to the primer by mixing 5 μ l template with 1 μ l of 2ng/ μ l M13 primer, 1 μ l 10x medium salt buffer, and 3 μ l H₂O. This was heated to 55°C for 1-2 h. To this was added 5 μ Ci [α -³²S]dATP \times S (supplied at 7000 μ Ci/mmol). 1 unit of Klenow fragment (1 μ l) was added, and mixed carefully. This mix was divided into four 2.5 μ l aliquots - A, C, G, and T. To each tube was added ^{2 μ l of} the relevant dNTP/ddNTP mix. These contained the following concentrations of nucleotides (given as μ M) in 10mM Tris-HCl pH7.5 (Hattori and Sakaki, 1988):

| | A | C | G | T |
|--|------|------|------|------|
| [α - ³² S]dATP \times S | 0.25 | 0.25 | 0.25 | 0.25 |
| dCTP | 88 | 8.25 | 83 | 83 |
| dGTP | 83 | 62.5 | 4.2 | 83 |
| dTTP | 83 | 62.5 | 83 | 6.25 |
| ddATP | 5 | - | - | - |
| ddCTP | - | 125 | - | - |
| ddGTP | - | - | 83 | - |
| ddTTP | - | - | - | 250 |

The reaction was started by spinning these mixes to the bottom of the tubes. The tubes were incubated at 30°C for 20 min. Then, 2 μ l of chase solution (0.5mM in each dNTP) were added to each, and the reaction continued for another 15 min. The reactions were stopped by adding 4 μ l 100% formamide, 0.01M

bromophenol blue to each tube.

II.K.ii Sequencing double-stranded plasmid DNA

DNA fragments were also sequenced in the pGEM vectors, using primers that annealed to the T7 and SP6 promoters. The plasmid was digested with an enzyme which linearized it downstream of the insert (Korneluk *et al.* 1985). It was incubated with 1 unit enzyme/ μ g DNA for 30 min only (to prevent nicking) then phenol/chloroform extracted, ethanol precipitated, and resuspended in water at 200ng/ μ l. 5 μ l DNA were then mixed with 2 μ l of the appropriate primer at 100ng/ μ l, 4 μ l water, and 1 μ l 10x medium salt buffer. This was boiled for 3 min, then cooled on ice. 1 μ l of 100mM DTT, 5 μ Ci (45 μ l) of [α -³²S]dATP, and 1 unit (1 μ l) Klenow fragment were added. This mixture was divided into four aliquots, and the sequencing reaction was set up and terminated as described above.

II.K.iii Sequencing gels

Sequencing reactions were analysed by electrophoresis in thin denaturing polyacrylamide gels. These contained 7M urea, 1xTBE, and the appropriate percentage of acrylamide [usually 6%(w/v), added from a stock solution of 38% acrylamide, 2% bisacrylamide]. Polymerization was catalysed by adding 0.1% TEMED and 0.6% ⁰ammonium persulphate. Gels were prerun in 1xTBE for 30 min at 42W. Meanwhile, the samples were denatured by being heated to 95°C for 5 min. The wells were washed out with buffer, the samples loaded using yellow Gilson tips, and the gel was run for 90 min at 42W. The best resolution of bands was obtained when a sharktooth comb (BRL) was used according to the manufacturer's instructions. After electrophoresis, the

back plate was removed carefully. The gel was fixed in 10%(v/v) acetic acid, transferred to Whatman 3MM paper, dried at 80°C and exposed at room temperature.

II.F. USE OF OLIGONUCLEOTIDES

II.F.i. Work up

Oligonucleotides synthesized on the Applied Biosystems synthesizer were received as 2ml eluates, containing about 500µg DNA, with the protecting groups still present. To remove the N-group benzoyl and isobutyryl group protection, an equal volume of 0.88 NH₃ solution was added to the eluate. This was transferred to polypropylene tubes and incubated at 55°C for 8 h. It was cooled on ice, transferred to a siliconized 25ml glass flask and the NH₃ removed in a dessicator, using a water pump. The oligonucleotide was then lyophilized overnight, and taken up in 1ml H₂O.

The next step was the removal of the 5' protecting dimethoxytrityl group. This was typically carried out on 200µl of the solution. 10µl triethanolamine was added to the remainder, and it was stored at -20°C in case subsequent analysis revealed that the oligonucleotide needed to be purified by HPLC - a process which requires the presence of the 5' protecting group. The 200µl aliquot was made 80% in acetic acid by adding 800µl glacial acetic acid, and this was incubated at room temperature for 20 min. 500µl water was then added, and the solution was ether extracted three times. Residual ether was blown off, and the oligonucleotide was lyophilized and taken up in 100µl water.

The oligonucleotide was quantitated by reading the A_{260} of an appropriate dilution, and characterized by kinasing in the presence of [γ - 32 P]ATP (see below) followed by electrophoresis on a 20X(w/v) acrylamide gel (see II.F.iii). All the oligonucleotides used in this project were sufficiently homogeneous not to need further purification.

Commercially produced oligonucleotides did not require these treatments.

II.F.ii Treatment with polynucleotide kinase

For most uses of oligonucleotides (e.g. ligation) 5' phosphate groups were added by treatment with T4 polynucleotide kinase as follows (see Maniatis et al. 1982, for more details): a 10 μ l reaction contained 2 μ g oligonucleotide, 50mM Tris-HCl pH8.0, 10mM MgCl₂, 10mM DTT, 5 μ Ci [γ - 32 P]ATP, and 5 units kinase. This was incubated at 37°C for 30 min, then 1 μ l of 10mM 'cold' ATP was added. The reaction was made up to 20 μ l by the addition of more Tris-HCl, MgCl₂ and DTT, then 5 units kinase were added and the mixture incubated at 37°C for a further 30 min. The kinase was then inactivated by heating the reaction to 70°C for 10 min.

II.F.iii Ligation

Where possible, i.e. if an oligonucleotide was palindromic or if two oligonucleotides were complementary, it was checked that the linkers would self-ligate. This was done by adding 1 μ l of kinased linkers to a reaction containing 50mM Tris-HCl pH7.6, 10mM MgCl₂, 10mM DTT, 1mM ATP. 2 units of T4 DNA ligase were added and the reaction was incubated at 4°C for about 4 h. Samples of kinase-treated ligated and unligated

linkers were then analysed by electrophoresis on thin non-denaturing 8%(w/v) polyacrylamide gels, made using a stock of 38% acrylamide, 2% bisacrylamide, and polymerized by the addition of 0.1% TEMED and 0.06% ammonium persulphate. The running buffer was 0.5xTBE, and the samples were loaded in the same solution as for agarose TBE gels (see II.B.i). The gel was prerun at 800V for 30 min, the samples were loaded, the gel run at 800V for about 2 h, and exposed wet.

For insertion into plasmids (see II.C.iv), the kinase-treated oligonucleotide was included in ligation mix at a range of concentrations from 0.5-10ng/ μ l - a vast molar excess over the plasmid.

II.G OLIGONUCLEOTIDE-DIRECTED MUTAGENESIS

The procedure used was based on that described by Zoller and Smith (1983), but with several modifications.

II.G.i Synthesis of mutant second strand

The DNA to be mutated was inserted into M13mp18 or mp19, and single-stranded template prepared as described above (II.E.i). Meanwhile, the oligonucleotide was treated with kinase in a 10 μ l reaction containing: 50pmol oligonucleotide, 50mM Tris-HCl pH8.0, 10mM MgCl₂, 5mM DTT, 1mM ATP and 5 units T4 polynucleotide kinase. This was incubated at 37°C for 30 min, then the enzyme was inactivated by heating to 70°C for 10 min. 5pmol kinased primer were annealed with 500ng single-stranded template, in a total of 5 μ l of 60mM Tris-HCl pH8.0, 60mM NaCl (Nakamaye and Eckstein, 1986).

This was heated to 70°C for 3 min, transferred to 37°C for 30 min, then cooled on ice. To this was added 3µl of solution containing: 25mM MgCl₂, 1.25mM each dNTP, 0.5mM ATP and 5mM DTT. Next, 1µl (1 unit) Klenow and 2µl (4 units) T4 DNA ligase were added. The reaction was incubated at 15°C overnight, then diluted to 100µl by the addition of TE.

II.G.ii Transformations

An overnight culture of *E. coli* BMH71:18 mut L cells (Kramer et al., 1984) was grown in L broth from a single colony. These cells are deficient in DNA repair. 2ml of this were used to inoculate 50ml of L broth, which was then incubated at 37°C until the A₅₅₀ was about 0.5. The cells were made competent as described above (II.E.i). A range of amounts, from 0.5-25ng, of the extension/ligation mix, were added to 300µl aliquots of cells, and transformations were carried out as described (II.E.i), except that no X-gal or IPTG were used, and 200µl of an exponential culture of non-mutant 71:18 cells were added. The cells were plated by adding 3ml molten B top agar (0.8% tryptone, 0.5% NaCl, 0.6% agar) to each tube and pouring onto a prewarmed B agar plate (0.8% tryptone, 0.5% NaCl, 1.5% agar). The plates were left to set, then incubated at 37°C overnight.

II.G.iii Screening of plaques

Plaques were gridded out onto duplicate fresh L broth plates, and grown up as colonies. The colonies were lifted from one of these plates onto a nitrocellulose filter. This was then placed sequentially on pieces of Whatman 3MM soaked in: 0.5M NaCl, for 5 min; 1M Tris-HCl pH 7.4, for 2 min (twice); 0.5M Tris-HCl pH 7.4, 1.5M NaCl, for 10 min. The nitrocellulose was air dried, then baked in a vacuum oven for 2 h at 80°C. It was then wetted in 6xSSC, and prehybridized in 30ml 10xDenhardt's, 6xSSC, 0.2% SDS at 67°C for 5 min. It was then quickly rinsed in 6xSSC.

Meanwhile, a radioactive probe was made in a 30 μ l reaction containing: 15pmol oligonucleotide, 50mM Tris-HCl pH 8.0, 10mM MgCl₂, 3mM DTT, 30 μ Ci [γ -³²P]ATP, and 2 units T4 polynucleotide kinase. This was incubated at 37°C for 30 min. 3ml 6xSSC were added and the mixture filtered through a Millipore filter unit. This was washed through with another 1ml of 6xSSC, which was then pooled with the rest of the probe.

The probe was added to the prehybridized filter, and incubated for 1h at room temperature. The filter was then washed three times for 3 min in 6xSSC at room temperature, wrapped in clingfilm, and exposed to X-ray film for 15 min. It was then washed four times for 1 min in 6xSSC at T_m-5. This was calculated for each oligo as follows:

$$T_m = 2(A+T)+4(C+G).$$

The filter was exposed again, then washed four times for 1 min at the T_m itself. At this temperature, the probe only remained hybridized to DNA derived from colonies containing the mutagenized sequence.

Bacteria containing the mutant DNA were picked from the master plate, diluted, and plated out again with fresh 71:18 cells, so that individual plaques could be isolated. These were grown up and analysed by direct sequencing of the single-stranded form (prepared as in II.E.i) or restriction enzyme digestion of the double-stranded form (prepared as in II.D.ii).

II.F EXTRACTION OF RNA

In all procedures involving RNA, great care was taken to avoid contamination with RNases: sterile distilled water was used throughout; solutions were autoclaved where possible; homogenizers were treated with chromic acid and baked at 200°C for 2 h; pipette tips and Eppendorf tubes were autoclaved; and disposable gloves were worn at all times.

II.F.1 Extraction of RNA from oocytes

This was carried out as described by Kressman *et al* (1978). In brief, the extraction buffer was 10mM Tris-HCl pH7.5, 1.5mM MgCl₂, 10mM NaCl, 1% SDS, 1mg/ml proteinase K. It was preincubated at 37°C for 20 min, to digest any RNases present. Up to 30 oocytes were then homogenized in 0.5ml buffer. Usually oocytes had been stored at -70°C, so buffer was added and they were homogenized in as short a time as possible. The homogenate was then incubated at room temperature for 20-25 min. After addition of NaCl to 0.3M, and EDTA to 10mM, it was extracted twice with an equal volume of phenol/chloroform (1:1), and once with chloroform alone. Finally, the RNA was ethanol precipitated, resuspended in 0.3M NaCl, and stored at -20°C as an ethanol precipitate. For

analysis, a volume equivalent to the required number of oocytes was removed, and the RNA recovered by centrifugation.

II.H.ii Extraction of influenza virus RNA

Three roller bottles, each containing approximately 10^8 BHK cells, were infected with influenza virus, and harvested 6 h later. This step was kindly carried out by Richard Rigg (University of Warwick). The RNA^{was} isolated from these cells by the guanidinium/caesium chloride method described in Maniatis et al (1982). Briefly, the cell pellets were taken up in a total volume of 18ml 8M guanidinium isothiocyanate, 5mM sodium citrate pH7.0, 0.1M 2-mercaptoethanol, 0.5% Sarkosyl. 1g CsCl was added per 2.5ml homogenate. The mixture was divided into 3ml aliquots which were layered onto 2ml cushions of 5.7M caesium chloride in 0.1M EDTA in MSE 5.5ml tubes. These were then centrifuged at 36000rpm for 16 h in an MSE 65, at 20°C. The resulting pellets of RNA were resuspended in a total of 3ml 10mM Tris-HCl pH7.4, 5mM EDTA, 1% SDS, then extracted once with an equal volume of chloroform/1-butanol(4:1), made 0.3M in sodium acetate, and finally precipitated with 2.2 volumes of ethanol.

After this ethanol precipitation, the RNA was reprecipitated by adding lithium chloride to 2.5M and storing at -20°C overnight - a procedure which does not precipitate DNA and low molecular weight RNA. The remaining RNA (approximately 2mg) was recovered by centrifugation, and poly(A)+ RNA purified by binding to a column of oligo-dT cellulose. This step was kindly carried out by Surinder Bhamra. The resulting poly(A)+ RNA was stored as an ethanol precipitate. When required, a portion was recovered by

centrifugation, and dissolved in water to a concentration of 100ng/ μ l.

II.1 IN VITRO TRANSCRIPTION

The methods used for in vitro transcription were based on the method described by Melton et al (1984).

II.1.1 Synthesis and processing of RNA

Preparation of template: Sequences to be transcribed were inserted into the vectors pGEM-1 and pGEM-2, which contain the promoters for both SP6 and T7 RNA polymerases separated by a multiple cloning site. This is in opposite orientation in the two vectors, so that by appropriate choice of vector and enzyme, both sense and antisense RNA could be transcribed from any sequence with either polymerase. In general, each plasmid was linearized with a restriction enzyme with a unique site in the polylinker downstream of the coding sequence from the appropriate promoter. The methods used for subcloning are described in sections II.B-D, and the enzymes used for linearization and transcription of each construct are specified in the text. DNA was recovered from the restriction digest by extraction with phenol/chloroform(1:1) once, then with chloroform once, followed by ethanol precipitation. The resulting pellet was taken up in water to give a final concentration of 0.5-1mg/ml.

Synthesis of capped transcripts: Briefly, the reactions were typically 50 μ l, and contained 50 μ g/ml DNA, 100 μ g/ml BSA, 0.5mM ATP, 0.5mM CTP, 0.5mM UTP, 50 μ M GTP, 0.5mM cap dinucleotide (see below), 40mM Tris-HCl pH7.5, 6mM MgCl₂, 2mM spermidine, 20mM DTT, 2000 units/ml RNasin, 300 units/ml SP6 or T7 polymerase. The reagents were added in that order, and at room temperature, to prevent precipitation of the DNA by spermidine. Usually, 1-5 μ Ci [α -³²P]UTP was also added, so that the efficiency of the reaction could be assessed (see below). Transcription occurred during incubation at 40°C for 1 h.

Choice of cap nucleotide: In most experiments, when capped RNA was required, 0.5M m⁷G(5')ppp(5')G was included in the reaction, and its incorporation was forced by using GTP at a concentration of only 50 μ M. However, in later experiments, the nucleotide G(5')ppp(5')G was used. The advantage of this procedure was that the transcription mix could be used without further purification, as the free cap nucleotide does not inhibit initiation of translation in oocytes or in the wheatgerm in vitro translation system.

Synthesis of uncapped RNA: Uncapped transcripts were synthesized in exactly the same way as capped transcripts, except that the cap nucleotide was omitted, and GTP was included at a concentration of 0.5M.

Processing of reactions: At the end of the incubation period, the reaction was made up to a volume of 50 μ l if necessary and a 2 μ l aliquot was removed for determination of incorporation (see below). To improve recovery, EDTA was then added to 10mM

and tRNA added to 200 μ g/ml. The salt, which was added next, was 0.7M ammonium acetate. This was preferred because the unincorporated nucleotides remained in the supernatant. The reaction was extracted once with phenol/chloroform(1:1), which was then back-extracted with 50 μ l H₂O. The aqueous phases were pooled, extracted twice with chloroform, then ethanol precipitated. After centrifugation, the RNA pellet was resuspended in 48 μ l H₂O, and a further 2 μ l aliquot was removed, this time to assess recovery of the RNA (II.I.iii).

To check that the RNA was full length, a small sample was also removed and analysed by electrophoresis on a formaldehyde agarose gel (see II.J.ii), followed by autoradiography. Capped RNA was then usually reprecipitated, and finally resuspended at a concentration of 100ng/ μ l for in vitro translation or microinjection into oocytes.

II.I.ii Synthesis of RNA probes

[³²P]-labelled probes were generally synthesized in 10 μ l reactions, containing a total of 50 μ Ci [α -³²P]UTP. The conditions were otherwise identical to those used for synthesis of capped RNA, except for the following: the cap nucleotide was omitted altogether; ATP, CTP and GTP were all added to a concentration of 0.5M; 10 μ M 'cold' UTP was also included to increase the yield of full length transcripts; finally, the amount of polymerase was also increased to 750 units/ml. After incubation at 40°C for 1 h, 40 μ l H₂O were added, and the reaction was then processed in exactly the same way as reactions including cap nucleotide (see above). Usually, after one ethanol precipitation, the probe was either used directly or further purified by electrophoresis on a

denaturing polyacrylamide gel.

II I iii Determination of incorporation

Although this method was primarily used for quantitation of synthetic RNA, it was also used whenever incorporation of radioactivity into nucleic acid had to be assessed. The 2 μ l samples removed from the reactions were diluted to 10 μ l, and two sets of duplicate 2 μ l samples were pipetted onto 1cm squares on Whatman DE81 paper. One set were washed five times in 0.15M Na₂HPO₄, for 2 min per wash, then rinsed in water, methanol, and finally acetone. They were allowed to dry, before being counted in Beckman EP scintillant. The unwashed samples were also counted to determine the total cpm in the samples. These figures gave the percentage incorporation, but they could also be used to estimate the amount of RNA synthesized. The maximum yield from a 50 μ l capped RNA synthesis was 3.6 μ g RNA, and this would give 10% incorporation of [³²P]UTP, since the concentration of the limiting nucleotide (GTP) was one tenth that of UTP. In practice, incorporation of more than 10% (implying yields of greater than 100%) were sometimes recorded. This was probably due to slight degradation of the cap nucleotide, allowing it to be incorporated instead of GTP.

II.J ANALYSIS OF RNA

II.J.i Selection of poly(A)+ RNA

The polyadenylation states both of endogenous oocyte and follicle cell RNA and of RNA transcribed after injection of DNA into oocytes were assessed by the ability of the RNA to bind to oligo-dT cellulose. The method used was based on the method described by Maniatis *et al* (1982), modified by R. W. Old. As a relatively large number of samples were analysed in each experiment, selection of poly(A)+ RNA was carried out using small batches (a few mg) of oligo-dT cellulose in Eppendorf tubes, rather than columns.

Firstly, the oligo-dT cellulose was equilibrated in loading buffer (20mM Tris-HCl pH7.6, 0.5M NaCl, 1mM EDTA, 0.1% SDS), and dispensed into 1.5 ml Eppendorf tubes so that it occupied a volume of about 30 μ l in each. The cellulose was lightly pelleted by centrifuging for 30s, and the excess loading buffer removed and replaced with the RNA (typically two oocyte equivalents) dissolved in 10 μ l H₂O and mixed with 0.4ml loading buffer. This was incubated at room temperature for 15 min, with intermittent shaking. The cellulose was pelleted and the loading buffer removed, and kept as the poly(A)- fraction. The oligo-dT cellulose together with bound RNA was washed five times with fresh loading buffer.

The poly(A)+ RNA was eluted by adding 0.5ml elution buffer (10mM Tris-HCl pH7.6, 1mM EDTA, 0.05% SDS) at 55°C, incubating at room temperature for about 5 min, then removing the buffer. This was repeated until all RNA had been eluted. In practice, this required only two washes with elution buffer. All fractions were centrifuged briefly to remove any remaining oligo-dT cellulose, then tRNA (10mg/ml) and NaCl

(0.3M) were added to the poly(A)+ fractions. The RNA was ethanol precipitated and analysed by northern blot or RNase mapping (see II.J.ii-iv).

II.J.ii Formaldehyde agarose gels

Formaldehyde agarose gels were used for RNA analysis in several different circumstances: after *in vitro* transcription, to check RNA was full length; after large scale isolation of RNA, to check the RNA was not degraded; and before transfer of RNA to nitrocellulose for analysis by northern blot. Usually gels were poured on glass plates 146mm x 200mm, but occasionally a 'minigel' apparatus was used. They contained 1% agarose, 1xMOPS buffer, and 15%(v/v) formaldehyde (added when the melted agarose had cooled below 60°C). RNA samples were typically 1-2 oocyte equivalents or about 100ng synthetic RNA, in 5µl H₂O. To each was added 15µl denaturing solution (made by adding 1 volume 10xMOPS buffer and 1.5 volumes formaldehyde to 5 volumes deionized formamide). The samples were heated to 60°C for 5 min and cooled on ice. 2µl 50% glycerol, 0.2% bromophenol blue were added to each, then they were loaded immediately. The gels were run submerged in 1xMOPS at 100V for 2-3 h.

Processing of gels: Gels containing only synthetic RNAs were fixed in 10%(v/v) acetic acid to remove free nucleotides, dried down, and exposed at -70°C with an intensifying screen. All other gels were stained to visualize the ribosomal RNA present in total and poly(A)- samples, to confirm that they were not degraded and that equal amounts had been loaded in all tracks. This was done by soaking each gel in 250ml

10X(w/v) glycine for 20 min to remove the formaldehyde, then adding ethidium bromide to 2 μ g/ml and staining for 10 min. After destaining for about 30 min, the 28S and 18S rRNA bands were clearly visible.

II.J.iii Northern blots

The method used for northern blots was based on the one described by Thomas (1980). The destained formaldehyde agarose gel (146mm x 200mm) was soaked in 20xSSC for 20 min and placed onto several thicknesses of Whatman 3MM paper, which were also saturated with 20xSSC. The exposed 3MM paper was covered with clingfilm. A sheet of nitrocellulose was placed onto the gel, and covered with another sheet of soaked 3MM paper, onto which tissues were stacked to a depth of about 3cm. Finally, a glass plate was placed on this, weighted, and the gel left to blot overnight. The next day, the nitrocellulose filter was removed, baked in a vacuum oven for 2 h, and stored at room temperature before use. It was prehybridized for 4-16 h at 42°C in a sealed plastic bag containing 12ml of prehybridization buffer [50% deionized formamide, 5xDenhardt's, 5xSSPE, 0.1% SDS, 100 μ g/ml sonicated, sheared, salmon sperm DNA, 10 μ g/ml poly(A)]. The bag was then opened, and excess prehybridization buffer removed. The probe (see below) was added in 4ml of the same buffer, sealed into the bag and incubated overnight. The filter was then washed, and exposed to X-ray film at -70°C with an intensifying screen. The incubation and washing conditions depended on the type of probe used.

Nick-translated probe: Fragments of DNA were nick-translated by a method based on that of Rigby et al. (1977). A 20 μ l reaction was set up containing about 100ng DNA, 1xNTB (see Maniatis et al. 1982), 1mM each of dATP, dGTP, and dTTP, 20 μ Ci [α - 32 P]dCTP, and 50ng/ml DNase I. 7.5 units DNA polymerase I were added and the reaction incubated at 15°C for 3 h. The DNA, of specific activity 10 7 -10 8 cpm/ μ g, was recovered by phenol/chloroform extraction and ethanol precipitation, and was sufficient for at least one large filter. It was denatured by being heated to 100°C for 5 min, and cooled rapidly on ice before being added to the hybridization buffer and incubated with the filter at 42°C overnight. The filter was then given four washes of 5 min in 2xSSC, 0.1% SDS at room temperature, followed by two washes of 15 min in 0.1xSSC, 0.1% SDS at 50°C.

Synthetic RNA probes: When RNA probes were used, the filters were prehybridized at 60°C (Zinn et al. 1983). The probe, typically 30-60ng of RNA of specific activity 3x10 8 cpm/ μ g, was denatured at 85°C for 5 min and added to the filter. Hybridization was at 60°C. The filter was then given four 15-30 min washes at 65°C in 2xSSC, 0.1% SDS, followed by one 30-60 min wash at 85°C in 0.2xSSC, 0.1% SDS.

Size markers: Radioactive size markers were produced by transcribing plasmids whose transcripts were of known size in the presence of [α - 32 P]UTP (see II.1.i). The plasmids were: pGEM-1 linearized with SphI (transcript 2.48kb); pGEM-1Vag1 (2.4kb); and pSP64H1 (1.65kb). The markers were loaded on formaldehyde gels along with the RNA samples to be analysed and blotted onto nitrocellulose.

II.J.iv RNase mapping

The RNase protection assay used was based on the method described by Krieg and Melton (1987).

Preparation of probe: The probe was synthesized as described above (see II.I.ii). After one ethanol precipitation, the pellet was dried and resuspended in 30 μ l of 80%(v/v) deionized formamide, 0.05% bromophenol blue. It was denatured at 85°C for 5 min, then loaded onto a 4% acrylamide sequencing gel (see II.E.iii). This was electrophoresed for about 90 min at 42W. The plates were separated and the wet gel covered with clingfilm. Orientation markers were added with radioactive ink, and the gel exposed to X-ray film for about 5 min. The full length probe was then cut out and eluted for 2-3 h at room temperature in 1ml elution buffer (0.5M ammonium acetate, 10mM magnesium acetate, 1mM EDTA, 0.1% SDS, 10 μ g/ml tRNA). Two small aliquots were then removed to check recovery; usually 70-80% was recovered. Two volumes of ethanol were then added to precipitate the probe.

Hybridization: Typically, samples were 1-2 oocyte equivalents of RNA stored in ethanol. A control containing 10 μ g tRNA was also included. To each was added 2x10⁶cpm of the probe in ethanol, and the sample and probe were coprecipitated by incubating at -70°C for 30-60 min, followed by centrifugation. The pellets were washed with 80% ethanol, dried, and resuspended in 3 μ l H₂O and 24 μ l deionized formamide. 3 μ l of 10x hybridization buffer (0.4M PIPES pH6.4, 4M NaCl, 10mM EDTA) were added and the samples were incubated overnight at

43°C.

Assay: RNase A to 20µg/ml and RNase T1 to 1µg/ml were added to RNase buffer (10mM Tris-HCl pH7.8, 0.3M NaCl, 5mM EDTA). 150µl of this were added to each sample, and they were incubated at 37°C for 15 min. 5µl of 10mg/ml proteinase K and 5µl 10% SDS were then added, and the samples incubated at 37°C for a further 20 min. They were then extracted once with phenol/chloroform(1:1). 5µg tRNA and 12µl 4M ammonium acetate were added and the RNA precipitated by adding 2 volumes of ethanol, and storing at -70°C for 30-60 min. The resulting RNA pellets were dried and resuspended in 10µl 80%(v/v) formamide, 0.05% bromophenol blue. The samples were denatured at 85°C for 5 min and half of each was loaded onto a 4%(w/v) acrylamide sequencing gel. This was electrophoresed at 42W for about 90 min. The gel was then fixed in 10%(v/v) acetic acid, dried down and exposed to X-ray film at -70°C with an intensifying screen.

II.K IN VITRO TRANSLATION AND ANALYSIS OF PROTEINS

II.K.i In vitro translation

Capped synthetic RNAs and natural poly(A)+ RNAs were translated in vitro, both to check they did encode the predicted polypeptide, and to provide size markers for comparison with their in vivo translation products. Usually, purified RNA solutions were used and these were translated in reticulocyte lysate. However, unpurified RNA in transcription mixes containing the capping dinucleotide GpppG was translated in wheatgerm, since the free GpppG inhibits translation in reticulocyte lysates.

Reticulocyte lysate: The message-dependent reticulocyte lysate used already contained haemin and creatine kinase (Jackson and Hunt, 1983). For assays, therefore, a mix of equal volumes of the following solutions was made: 2M KCl / 10mM MgCl₂; 200mM creatine phosphate; 2mM all amino acids except methionine; 15μCi/μl [³⁵S]methionine. One volume of this was added to 4 volumes of newly thawed reticulocyte lysate, on ice. This mixture was divided into 10μl aliquots, and the RNA added. As almost all the RNA samples used contained only one major species, the concentration was adjusted to 100ng/μl and 1μl was added to each translation. After incubation at 30°C for 1 h, the translation reaction was stopped by returning samples to ice.

Wheatgerm: Assay mix was set up according to the method of Anderson *et al* (1983), so that the final composition was: 20mM HEPES pH7.6, 1mM ATP, 8mM creatine phosphate, 40μg/ml creatine kinase, 30μg/ml spermine, 2mM DTT, 20μM GTP, 25μM each amino acid except methionine, 2.5mM magnesium acetate, 120mM potassium acetate, and 0.75μCi/ml [³⁵S]methionine. It was divided into 10μl aliquots, to each of which 0.5μl transcription mix was added. The samples were then incubated at 30°C for 90 min. The reactions were stopped by returning the tubes to ice.

Analysis: When samples contained ³²P-labelled RNA, they were treated by addition of RNase A to a concentration of 50μg/ml and incubated at room temperature for about 10 min before further analysis. In general, one 2μl aliquot was used to assess incorporation of [³⁵S]methionine (see II.K.ii), and

another for analysis by SDS-PAGE (see II.L). When necessary, immunoprecipitation was performed on a further 2 μ l aliquot (II.K.iii).

II.K.ii Determination of incorporation of radioactive amino acid

Incorporation of [³⁵S]methionine into polypeptides was assessed by the method described by Clemens (1984). Duplicate 2 μ l aliquots (usually produced by diluting a single 2 μ l sample) of homogenates or in vitro translation mixes were pipetted onto 1cm squares of Whatman no. 1 filter paper. One set of samples was washed successively for 15 min each in ice-cold 5%(w/v) TCA, 5% TCA containing 3%(w/v) casamino acids at 90°C, and 5% TCA at room temperature. Finally they were rinsed in absolute ethanol then acetone and allowed to dry. Incorporated label was determined by adding Beckman EP scintillant and counting these TCA washed samples in a Beckman liquid scintillation counter. The untreated samples were counted to determine the total amount of radioactivity present.

II.K.iii Immunoprecipitation

1-100 μ l aliquots of in vitro translation mix or oocyte homogenate were added to 0.45ml of detergent mix buffer (DMB; 100mM Tris-HCl pH 7.8, 1% Triton-X100, 0.5% SDS, 5mM MgCl₂, 100mM KCl, 1% deoxycholate, 1mM methionine, 1mM PMSF), containing the appropriate antibody. For most of the preparations, 1 μ l was used per sample; however, for the rabbit anti-influenza virus, 5 μ l were used per sample. The samples were incubated on ice for 30 min, after which 80 μ l of a

10%(v/v) suspension of formaldehyde-treated envelopes of Staphylococcus aureus (Kessler, 1975; prepared by Linda Tabe, University of Warwick) were added to each. They were left shaking at 4°C for at least 2 h - usually overnight. The envelopes and bound antigen/antibody complexes were pelleted by spinning in a microfuge for 20 s and washed twice in fresh DMB. They were then resuspended in sample buffer, in which the complexes dissociate, and prepared for analysis by SDS-PAGE (see below).

II.L ELECTROPHORESIS OF PROTEINS

Radiolabelled proteins synthesized either by in vitro translation systems or by oocytes were generally analysed by one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins of interest were often immunoprecipitated before analysis, but total translation products were also analysed. In some cases, where immunoprecipitation was not possible, proteins were analysed by two-dimensional electrophoresis. Electrophoresis of proteins is discussed in detail in Hames and Rickwood (1981), and I give only a brief outline here.

II.L.i One dimensional SDS-PAGE

Preparation of samples: Two different methods were used. In the first, immunoprecipitate pellets or 1-10 μ l of in vitro translation mix or total oocyte homogenate were taken up in Sample Buffer 1 [0.2M Tris-HCl pH8.8, 2%(w/v) SDS, 5mM EDTA, 1M sucrose, 0.01% bromophenol blue, 3.3mM DTT], to a volume of 30 μ l. Samples were heated to 100°C for 3 min, allowed to cool, then alkylated by the addition of iodoacetic acid to 70mM.

After incubation at room temperature for approximately 15 min, they were spun in a microfuge for 1 min before being loaded onto the gel. When the homogenate or translation mix contained 32 P-labelled synthetic RNA, the samples were treated with RNase A (50 μ g/ml, room temperature, 10 min) before preparation.

In later experiments, a second method gave better resolution of proteins: 10 μ l of homogenate was added to 10 μ l of 2X Sample Buffer 2 [0.125M Tris-HCl pH6.8, 4%(w/v) SDS, 20%(v/v) glycerol, 0.01% bromophenol blue, 5%(v/v) 2-mercaptoethanol]. Samples were heated to 100°C for 3 min and spun in a microfuge for 1 min before being loaded onto the gel. Immunoprecipitate pellets or samples of 1 μ l translation mix were taken up in 20 μ l 1X Sample Buffer 2, then treated in the same way.

In both cases half of each sample (corresponding to one eighth of an oocyte) was usually loaded, and the remainder stored at -20°C. When a further aliquot of the same sample was required, it was thawed, vortexed, spun for 1 min, and loaded onto the gel.

Markers: The markers used were a commercial preparation of 14 C-labelled methylated proteins: lysozyme, 14.3kD; carbonic anhydrase, 30kD; ovalbumin, 46kD; BSA, 69kD; phosphorylase, 92.5kD; myosin, 200kD. 10 μ g of cytochrome c was also loaded onto each gel. This gave a visual indication of the resolution during electrophoresis.

Electrophoresis: Slab gels were run using a discontinuous buffer system based on the method of Laemmli (1970). Resolving gels were cast containing the appropriate percentage acrylamide, 0.375M Tris-HCl pH8.8, 0.1% SDS, 0.04% ammonium persulphate, and 0.06% TEMED. A stock solution of 30%(w/v) acrylamide, 0.825% bisacrylamide was used. All the gels described in this thesis were 10% or 12.5% acrylamide. The stacking gel always had the same composition: 5%(w/v) acrylamide, 62.5mM Tris-HCl pH6.8, 0.1% SDS, 0.08% ammonium persulphate, 0.06% TEMED.

After loading of the samples, gels were usually electrophoresed at 20mA per gel until the dye had entered the resolving gel, when the current was increased to 30mA per gel. Occasionally, however, gels were run at a lower current for a longer period. The electrophoresis buffer was 25mM Trizma, 0.192M glycine, 0.1% SDS.

Fixation and Fluorography: Gels were fixed for 1-48 h in 45%(v/v) methanol, 10%(v/v) acetic acid. They were then fluorographed either by the method of Bonner and Laskey (1974) as described in Hames and Rickworth (1981), or by treatment with the commercial preparation 'En³Hance'. However, 'En³Hance' was used only with 10% acrylamide gels, since higher percentage gels tended to crack while drying down after this treatment. Dried, fluorographed gels were autoradiographed by exposure to X-ray sensitive film at -70°C.

II.L.ii Two-Dimensional Gel Electrophoresis

Two-dimensional analysis of proteins was carried out by a method based on that of O'Farrell (1975).

Preparation of samples: Oocytes were usually homogenized in homogenization buffer (see II.M.ii), so that the same samples could be analysed on both one- and two-dimensional gels. For two-dimensional gels, one volume of homogenate was mixed with two volumes of lysis buffer [9.5M urea, 2%(v/v) NP-40, 5%(v/v) 2-mercaptoethanol, 1.6%(v/v) ampholines pH3.5-10, 3.4%(v/v) ampholines pH5-7]. Usually, 50 μ l were loaded per tube gel, corresponding to just under half an oocyte.

Isoelectric focusing: First dimension, isoelectric focusing gels 11cm long were cast in 13cm lengths of 2.5mm diameter glass tubing. The composition of the gels was: 4%(w/v) acrylamide (from a stock of 28.4% acrylamide, 1.6% bisacrylamide), 9.17M urea, 2%(v/v) NP-40, 1.7%(v/v) ampholines pH3.5-10, 3.3%(v/v) ampholines pH5-7, 0.012% ammonium persulphate and 0.07% TEMED. The gels were overlaid with 8M urea during polymerization, after which it was replaced with 10 μ l lysis buffer.

The gels were pre-run at 200V for 15 min, 300V for 30 min and 400V for 30 min, using 20mM NaOH as cathode buffer, and 10mM orthophosphoric acid as anode buffer. The lysis buffer and NaOH were then removed from the tops of the gels, and the samples loaded. They were overlaid with 10 μ l of 9.17M urea, 0.083% ampholines pH 3.5-10, and 0.167% ampholines pH 5-7. The gels were then run at 400V for about 18 h.

After electrophoresis, the gels were removed from the tubing and transferred to equilibration buffer [70mM Tris-HCl pH6.8, 2.2%(v/v) SDS, 5.6%(v/v) 2-mercaptoethanol]. The gels were either loaded onto the second dimension after 30-60 min in equilibration buffer, or frozen at -70°C until second dimension gels could be run.

Determination of pH gradient: A 'blank' tube gel was always included when isoelectric focusing gels were run. After electrophoresis, this was removed from its glass tubing, cut into 1cm lengths, and each piece was incubated with 2ml distilled water for 20 min at room temperature. The pH gradient was then determined by measuring the pH of each solution.

SDS-polyacrylamide gel electrophoresis: For the second dimension, gel recipes were exactly the same as for one-dimensional gels (see II.L.i). The resolving gel was first poured to a depth 2cm less than the height of the plates, then the stacking gel was poured to reach the top of the plates. Once this had set, the IEF gel was placed carefully onto it, and sealed into position with a melted mixture of 1% agarose, 0.35% SDS, 70mM Tris-HCl pH6.8 and 0.01% bromophenol blue. The gels were usually run at 20mA through the stacking gel and 30mA through the resolving gel, but occasionally they were run at low current (5-10mA) overnight. They were then fixed and fluorographed as normal.

II.M MICROINJECTION AND ANALYSIS OF OOCYTES

The frogs, Xenopus laevis and Xenopus borealis, were maintained by Mrs Celia Kwasnik, as described by Colman (1984b). Most other procedures involving frogs and oocytes were also carried out by methods described by Colman (1984a and b), and he gives full practical details, so I give only a brief outline here.

II.M.i Preparation and microinjection of oocytes

Oocytes were obtained by removing portions of ovary from frogs anaesthetized by immersion in 0.2% ethyl m-aminobenzoate (MS222). The oocytes were washed thoroughly and stored in modified Barths' saline (MBS; 88mM NaCl, 1mM KCl, 2.4mM NaHCO₃, 15mM Tris-HCl pH7.6, 0.3mM CaNO₃, 0.41mM CaCl₂, 0.82mM MgSO₄) containing penicillin at 8mg/ml and streptomycin at 10mg/ml. After being separated manually, they were incubated at about 20°C for not longer than 24 h before microinjection. Typically, 20nl DNA at 0.2mg/ml were injected into the germinal vesicle or 50nl RNA at 0.1mg/ml were injected into the cytoplasm (Colman, 1984b). The oocytes were then incubated at 20°C for a further 24 h before analysis of RNA content or protein synthesis. Operations on frogs and microinjection of oocytes were kindly performed by Dr Alan Colman.

II.M.ii Incubation and processing of oocytes

Protein samples: Oocytes were labelled by incubation in 5 μ l/oocyte of MBS containing [³⁵S]methionine at 1mCi/ml, unless indicated otherwise in the figure legend. If the oocytes had been defolliculated before labelling, 5%(v/v) dialysed fetal calf serum was also added to the medium. Heat shocked samples were transferred to 35°C 15-20 min before addition of the radioactive MBS, to allow the heat shock response to begin. They were then incubated at this temperature for a further 2 h in the presence of the label. Non-heat-shocked samples were labelled for 2 h at 20°C.

After the incubation period, the oocytes were washed in fresh MBS, and placed on ice. Dead or damaged oocytes were removed and the remainder were defolliculated, if necessary. They were then usually frozen on dry ice before homogenisation in 40 μ l/oocyte of 100mM NaCl, 1% Triton-X100, 1mM PMSF, 20mM Tris-HCl pH7.6. Yolk platelets were pelleted by centrifugation for 1 min in a microfuge, and homogenates stored at -20°C before analysis by immunoprecipitation and/or SDS-PAGE.

RNA samples: When the RNA content of the oocytes was to be analysed, they were incubated at the appropriate temperature, defolliculated if necessary, then frozen on dry ice and stored at -70°C before extraction of the RNA. When the nucleus and cytoplasm were to be analysed separately, the oocytes were heated to 100°C in MBS for 3 min, chilled on ice, then broken into halves with forceps to release the nucleus (Georgiev *et al.* 1984).

II.M.iii Estimation of follicle cell numbers

Somatic cells on the oocytes were visualized by staining with MBS containing 1µg/ml Hoescht dye 33258, then viewed under a fluorescent microscope. To estimate the number of somatic cells, each oocyte was photographed twice - once with the equator in focus, and once with the pole in focus. It was assumed the oocyte was a sphere of radius (r), determined from the first photograph. The cells (n) in a circle of radius (a) around the pole were counted, from the second photograph, and this was used to calculate the number of cells (N) on the whole oocyte as follows:

$$\text{Height of zone } h = r - x$$

$$(\text{where } x^2 = r^2 - a^2)$$

$$\text{Surface area of zone} = 2 \pi r h$$

$$\text{Surface area of sphere} = 4 \pi r^2$$

$$\begin{aligned} \text{Total number of cells } N &= 4 \pi r^2 n / 2 \pi r h \\ &= 2 r n / h \end{aligned}$$

II.M.iv Defolliculation

Oocytes were incubated for 10 min at room temperature in 0.25% collagenase contained in Barths' A solution (88mM NaCl, 1mM KCl, 2.4mM NaHCO₃, 15mM Tris-HCl pH7.6). The theca was then removed by manual dissection with fine watchmaker's forceps. After a further 5 min in collagenase, the oocytes were transferred to stripping solution (0.2M K-aspartate, 20mM KCl, 1mM MgCl₂, 10mM EGTA, 10mM HEPES pH7.2; Sakmann *et al.* 1985), for 10 min. This separated the vitelline layer from the plasma membrane and allowed it to be removed by manual dissection, along with any remaining follicle cells. If they were not to be frozen or homogenized immediately,

defolliculated oocytes were incubated in 5%(v/v) dialysed fetal calf serum in MBS. When oocytes were defolliculated after radioactive labelling, 1mM methionine was added to all solutions to reduce incorporation of [³⁵S]methionine into protein. In some of the experiments described here, the defolliculation was kindly carried out by Alan Colman and John Shuttleworth.

CHAPTER III: RESULTS AND DISCUSSION

SYNTHESIS OF hsp70 IN OOCYTES AND FOLLICULAR CELLS

III.A Introduction

The original aim of this project was to attempt to identify the sequences in Xenopus hsp70 mRNA responsible for allowing it to be preferentially translated during heat shock and masked at normal temperatures in oocytes, by monitoring the expression of systematically mutated hsp70 genes. The conclusions of King and Davis (1987; see I.B.v) that oocytes do not have a heat shock response, if correct, would obviously have profound implications for the feasibility of this approach. It therefore became an urgent priority to carry out a rigorous study to determine the extent of the contribution of the follicle cells to the synthesis of hsp70.

Since a possible explanation for the discrepancies between the results of King and Davis (1987) and Biens and Gurdon (1982) would be that the defolliculation method used by Biens and Gurdon did not in fact remove all the follicle cells, the first step was to devise a way of completely and reliably removing all cells adhering to oocytes. Next, the RNA content of oocytes both with and without their somatic cells was estimated before and after heat shock, to assess whether either cell type had the potential to make hsp70. Finally, the patterns of protein synthesis in both cell types were re-examined.

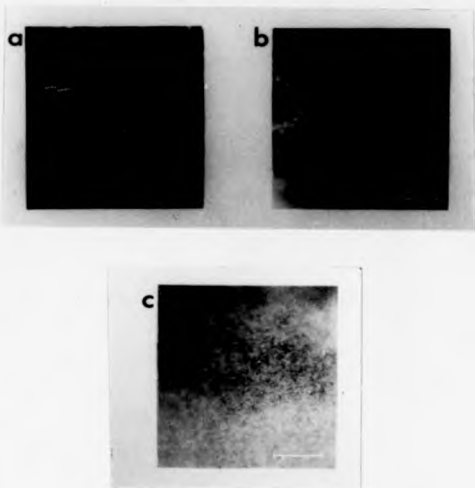


FIG. III.1 OOCYTES AT VARIOUS STAGES OF DEFOLLICULATION.

Oocytes were stained with Hoechst 33258 in MBS (II.W.4ii), then photographed under UV and visible light: (a) a folliculated oocyte; (b) an oocyte with islands of follicle cells remaining after removal of the theca; (c) a completely defolliculated oocyte.

III.B Defolliculation Procedure

Xenopus oocytes are, in fact, surrounded by two layers of somatic cells - an outer thecal layer containing blood vessels and fibroblasts and an inner layer of follicle cells (Dumont and Brummet, 1978). These cells can be visualized by staining an untreated oocyte with Hoescht dye and viewing under a fluorescent microscope. Figure III.1a shows such an untreated (or 'folliculated') oocyte. The dark band in the centre is a blood vessel. Nuclei in the thecal layer have a distinctive 'comma' shape and are more sparsely distributed than those in the inner follicle cell layer.

An estimate was made of the number of follicle cells present by photographing stained oocytes, counting the cells in a small circular area of the photograph and integrating this number over the surface area of the oocyte (see II.M.III). For fully grown oocytes, the mean number of cells was 8000 ± 1000 (data not shown). The number of thecal cells was lower and more variable.

A variety of different methods of removing these cells were tested, but the following procedure proved to be the quickest and most effective (for a full description, see II.M.iv) and was used for all defolliculation operations described in this thesis, unless otherwise stated. Oocytes were given a brief treatment with collagenase before removal of the thecal layer by manual dissection. After a second collagenase treatment, the oocytes were incubated in a hypertonic medium in which the vitelline envelope separated from the plasma membrane. It was then removed manually, together with any cells in it. Because these completely defolliculated oocytes were very fragile, they were always

inoculated in modified Barths' solution (MBS) containing 5% fetal calf serum, unless they were to be homogenized immediately. Although they tended to flatten, they did not die and were capable of synthesizing protein.

Removal of the somatic cells was monitored by staining with Hoechst dye. This technique had the advantage that it did not require the sample to be fixed, so that protein synthesis could subsequently be examined in the stained oocytes themselves, rather than in a parallel sample. Figure III.1b demonstrates that, although many follicle cells were removed with the theca, some also remained embedded in the vitelline membrane. The proportion lost at this stage varied considerably between batches of oocytes, but remaining cells were invariably removed with the vitelline membrane (fig. III.1c). It was therefore crucial to remove both the theca and the vitelline membrane to ensure that no follicle cells were left attached to the oocyte.

In general, unless making a specific distinction between the two layers, I will refer to all the somatic cells on an oocyte as, simply, follicle cells.

III.C hsp70 RNA content of oocytes and follicle cells

The experiments described in this section were carried out by Dr John Shuttleworth.

If the synthesis of hsp70 by Xenopus oocytes is indeed regulated entirely at the level of translation, then non-heat-shocked oocytes must obviously contain levels of its mRNA sufficient to account for the rate of production observed on heat shock. By the same token, if, as King and Davis (1987)

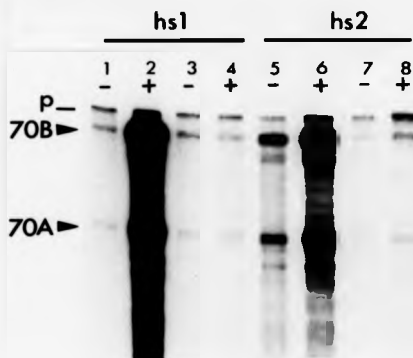


FIG. III.2 LEVELS OF hsp70A AND hsp70B TRANSCRIPTS IN OOCYTES AND FOLLICLE CELLS.

Total RNA extracted from oocytes was analysed by RNase protection (II.J.iv) using the 506-nucleotide hsp70B probe. RNA was analysed from folliculated oocytes (tracks 1 and 2) and oocytes defolliculated immediately before homogenization (tracks 3 and 4), both before (-) and after (+) a single 2h heat shock at 35°C. A further batch of heat shocked oocytes was allowed to recover for 24h at 20°C, and the same analysis was performed on folliculated (tracks 5 and 6) and defolliculated (tracks 7 and 8) oocytes before (-) and after (+) a second heat shock at 35°C for 2h. RNA equivalent to one oocyte was analysed in each case.

The levels of hsp70A and hsp70B transcripts are indicated by the levels of the 368- and 478-nucleotide protected fragments respectively (marked with arrows); residual full length probe is also indicated (p).

suggest, the hsp70 is actually produced in the follicle cells by the 'classical' mechanism (see I.B.v) then a heat shock must induce hsp70 gene transcription in these cells.

To investigate whether either of these conditions holds, the levels of transcripts in folliculated and defolliculated oocytes were determined before and after a two hour heat shock, and the results are illustrated in figure III.2. A heat shock temperature of 35°C was chosen for all the experiments described in this thesis, because it gave maximum induction of the heat shock response, and good survival of the oocytes. If incubated at temperatures above 36°C, almost all oocytes died within two hours.

The RNA was analysed by an RNase protection (II.J.iv) assay which distinguished two forms of hsp70 message, which are transcribed from different genes (hsp70A and hsp70B; Bienz, 1984b). When the 506-nucleotide radioactive probe was annealed to hsp70 RNA and digested with RNase, hsp70B generated a protected fragment of 478 nucleotides, and hsp70A generated a fragment of 368 nucleotides. The evidence for this and the details of the assay itself are discussed in Chapter IV.B.

The non-heat-shocked oocytes did contain clearly detectable levels of both hsp70A and hsp70B mRNA (tracks 1 and 3). Since the level was identical in both folliculated and defolliculated oocytes, the transcripts must have been in the oocytes themselves, rather than in the follicle cells. The amount of hsp70 mRNA present was estimated by titrating various amounts of synthetic sense hsp70 RNA (from 2.5 to 5000 pg) in the RNase protection assay and comparing the counts eluted from the gel for each sample (data not shown). Up to 6

10^6 copies (10 pg) of hsp70A and 2×10^6 copies (3 pg) of hsp70B were found per oocyte. These values are similar to the total of 3×10^6 copies per oocyte quoted by Bienz (1984a). However, it should be noted that, curiously, we found considerable variation between frogs in both the absolute and relative amounts of hsp70A and hsp70B mRNA, for reasons which are not known (data not shown). In most frogs tested, the levels were around 5- to 10-fold less than the maximum, and in some frogs the oocyte expression of hsp70A was greater than that of hsp70B, whereas in others the reverse was true. This effect was invariably paralleled in the accompanying follicle cells during heat shock.

Heat shock did not result in a detectable increase in the hsp70 mRNA in the oocyte itself (track 4), and RNA in the oocyte was not degraded after heat shock (track 7). In contrast, there was a dramatic increase in the amount of hsp70 mRNA in folliculated oocytes (track 2), attributable to induction of transcription of follicle cell genes. Up to 4.2×10^7 hsp70A and 3.4×10^7 hsp70B transcripts were synthesized per oocyte complement of follicle cells - nearly 10 times the level of transcripts present within the oocyte. This represents a transcription rate of 7×10^2 transcripts per gene per hour, assuming 6000 follicle cells per oocyte (see section III.B) and, since *Xenopus laevis* is tetraploid, four hsp70A and four hsp70B genes per nucleus. In the 24 hours following return to normal temperatures (track 5) 70% of these transcripts were degraded, but transcription was induced again by a subsequent heat shock (track 6).

These results suggest that the follicle cells mount a 'classical' type of heat shock response. Since much more RNA is induced in these cells than is present in oocytes, it seemed likely that they would contribute most of the hsp70 synthesized by folliculated oocytes. However, there is a small amount of stable hsp70 mRNA in oocytes, a finding consistent with the conclusions of Biens and Gurdon (1982), that oocytes contain 'masked' hsp70 mRNA which is translated only on heat shock. The results described here say nothing about the translational status of these messages, though, so it was necessary to complement the RNA studies with analysis of protein synthesis.

III.D Patterns of protein synthesis in folliculated and defolliculated oocytes.

Two distinct patterns of protein synthesis in Xenopus oocytes during heat shock have been reported in the literature (see I.B.v). In the first, described by Biens and Gurdon (1982), hsp70 is the only heat shock protein synthesized by folliculated oocytes. This pattern was observed in about 70% of our experiments. However, in the other 30% of experiments, another pattern was observed: translation of several proteins was enhanced by heat shock. This pattern corresponded with that described by Browder et al (1987), and is discussed in detail in section III.F.

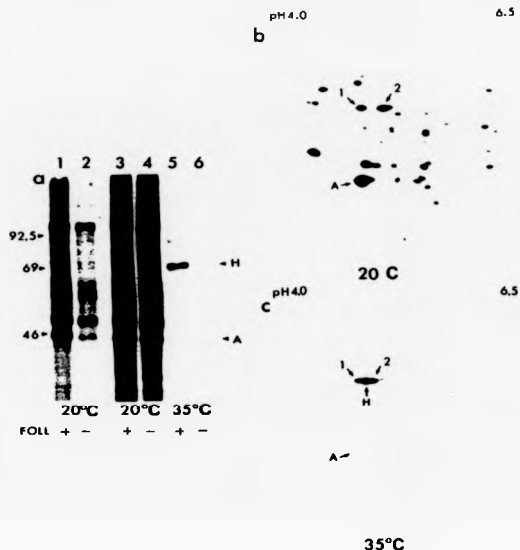


FIG. 111.3 NORMAL PATTERNS OF PROTEIN SYNTHESIS AT 20°C AND DURING HEAT SHOCK.

Oocytes complete with follicle cells were incubated in MBS containing $1\mu\text{Ci}/\text{ml}$ [^{35}S]methionine for 2h at 20°C (tracks 1-4) or 35°C (tracks 5 and 6). The samples in tracks 2, 4 and 6 were defolliculated before analysis on 10% polyacrylamide gels; the remainder were analysed with their follicle cells still present (tracks 1, 3 and 5). Tracks 1 and 2 are a shorter autoradiographic exposure of tracks 3 and 4. Folliculated oocytes labelled at 20°C (b) and 35°C (c) were also analysed by two-dimensional electrophoresis.

Sizes of molecular weight markers are given in kD; actin is indicated A; hsp70, H; proteins 1 and 2 are discussed in 11.D.i.

III.D.1 Folliculated oocytes

Figure III.3 shows representative examples of normal protein profiles of folliculated oocytes incubated for two hours in the presence of [35 S]methionine at 20°C and 35°C, analysed on both one- and two-dimensional gels (II.L and II.M). Comparisons between samples were always made of equal 'oocyte equivalents': in general, the equivalent of one eighth of an oocyte was loaded per track on one-dimensional gels, and half an oocyte was loaded on each two-dimensional gel. On heat shock, the overall rate of protein synthesis fell dramatically (by up to 90%, data not shown), and hsp70 (marked H) was the major protein synthesized. A band corresponding to protein of 70 kD is seen in the 20°C sample on the one-dimensional gel (fig. III.3a, track 3 and, in a shorter exposure, track 1), but on the two-dimensional gels this band resolved into two spots which migrated very slightly slower than hsp70 (fig. III.3b and c, proteins 1 and 2). The synthesis of hsp70 itself was not detectable at 20°C. The mobilities of proteins 1 and 2 are so similar to that of hsp70, that it seems likely that they are the related heat shock cognates (Craig *et al.* 1983; see I.B.1).

Surprisingly, the translation of these two proteins appears to be maintained at 35°C in this experiment. This selective translation was not always observed. There was also variation between batches of oocytes in the exact pattern of spots near those corresponding to the putative heat shock cognates 1 and 2. In some cases additional spots were seen, and these were occasionally present in samples of defolliculated oocytes. As was observed for proteins 1 and 2, synthesis of these proteins was often maintained during heat

shock. They were distinguishable from the major follicle cell hsp70, which migrates between proteins 1 and 2 in the isoelectric focussing dimension, and slightly ahead of them in the SDS-PAGE dimension (fig. III.3c).

Many other variations in the patterns of protein synthesis were observed between and within different batches of oocytes, and these sometimes made interpretation of the results difficult. Certain proteins were much more prominent in some samples, and there was often great variation in the overall level of incorporation and the reduction in rate of protein synthesis on heat shock. A comparison of the 'ideal' results shown in figure III.3 with those shown in figures III.5 and V.3 illustrates some of these problems.

III.D.ii Oocytes defolliculated after labelling

When the follicle cells were removed after incubation in [³⁵S]methionine, the picture was very different. Clearly, follicle cells do make a significant contribution to overall protein synthesis, since there was less radioactive protein in the defolliculated (fig. III.3, tracks 4 and 6) than folliculated samples (tracks 3 and 5). Estimates based on acid precipitation of incorporated [³⁵S]methionine from oocyte homogenates from several experiments were that follicle cells can contribute up to about 50% of the overall protein synthesis at 20°C and up to 80% at 35°C (data not shown).

Most importantly, though, all of the labelled hsp70 was removed with the follicle cells (track 6). In the original autoradiograph, a very faint band is visible at the position of hsp70, although this is not seen in the photograph. However, two-dimensional analysis of defolliculated,

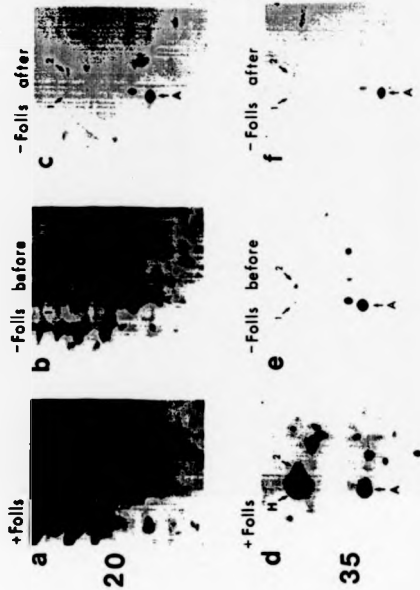
FIG. III.4 TWO-DIMENSIONAL

ANALYSIS OF PROTEIN SYNTHESIS IN
OOCYTES WITH AND WITHOUT ATTACHED
FOLLICLE CELLS.

Oocytes were incubated in MBS
containing [³⁵S]methionine for 2h at 20°C
(a-c) or 35°C (d-f), then

analysed by two-dimensional
electrophoresis: (a) and (d) show
follicululated oocytes; (b) and
(e), oocytes defolliculated
before labelling; (c) and (f),
oocytes defolliculated after
labelling.

Actin is indicated A; hsp70, H;
proteins 1 and 2 are discussed in
III.D.1.



heat-shocked oocytes reveals that this is due to proteins 1 and 2, not to hsp70 itself (see fig. III.4, panels c and f). These results indicate that hsp70 is not synthesized in oocytes in detectable amounts, and that it is not transported into oocytes from follicle cells.

III.D.iii Oocytes defolliculated before labelling

In the experiment illustrated in figure III.4, different samples of oocytes were also defolliculated before being radioactively labelled and heat shocked. The reasons for this were twofold. Firstly, it seemed possible that, when follicle cells were present, radioactively labelled oocyte proteins might be under-represented, since [35 S]methionine would be taken up and equilibrated with the cytoplasmic pool of amino acids more quickly by the follicle cells than by the oocytes. Equilibration takes 15 to 30 minutes in oocytes, even when the radioactive amino acid is injected (Shih *et al.* 1978). Secondly, it was also possible that the loss of follicle cells itself might prevent oocytes from mounting a heat shock response. Indeed, Moor and Osborn (1983) have demonstrated that signals from follicle cells can regulate protein synthesis in mammalian oocytes, at least. They found that the removal of follicle cells from mammalian oocytes resulted in a dramatic and specific reduction in actin synthesis within the oocytes.

The results shown in figure III.4 indicate that neither of these possibilities affects the interpretation of the results of figure III.3. Although there was a generally lower level of incorporation of [35 S]methionine into protein in defolliculated oocytes at both temperatures (panels b and e),

there was no specific reduction in actin synthesis, so the regulation found in mammals evidently has no counterpart in amphibians. Moreover, although in this experiment the level of incorporation of label was greater in oocytes defolliculated before labelling (panels b and e) than in those defolliculated after labelling (panels c and f), the situation was reversed in other experiments. This indicates that, even though equilibration of label may take place faster in follicle cells than in oocytes, their presence probably does not itself affect the rate of equilibration in oocytes.

No hsp70 was detectable in heat shocked oocytes whether the follicle cells were removed before or after heat shock (panels e and f). The follicle cells therefore do not affect the oocytes' heat shock response - which apparently consists only of a reduction in their overall rate of protein synthesis, with no accompanying induction of the synthesis of specific proteins.

III.E Effect of partial removal of follicle cells on protein synthesis

In early experiments, several different methods of removing follicle cells were tested, before the optimum method was devised. Although these were not developed as routine methods, they did demonstrate some important points about the heat shock response of Xenopus oocytes.

In one such experiment, oocytes were incubated in collagenase for about two hours and the theca was removed by manual dissection. Some of the oocytes were then incubated at 20°C overnight in MBS, during which time many of the remaining follicle cells simply fell off into the medium. Others were

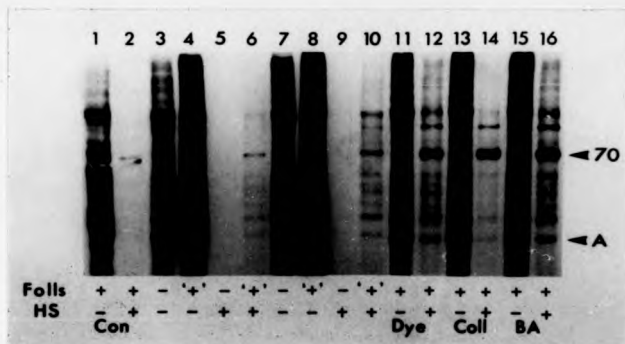


FIG. III.5 PARTIAL DEFOLLICULATION OF OOCYTES. Oocytes were incubated for 2h in MBS containing 1mCi/ml [^{35}S]methionine at either 20°C (HS-) or 35°C (HS+), and analysed on 10% SDS-polyacrylamide gels. Tracks 3-6: oocytes treated before labelling by incubation in 0.25% collagenase in Barth's A solution, followed by manual removal of the theca and overnight incubation in 0.25% sheep hyaluronidase in MBS - tracks 3 and 5 show oocytes with no attached follicle cells; tracks 4 and 6, oocytes with some cells remaining. Tracks 7-10: oocytes treated as for oocytes shown in tracks 3-6, except that the overnight incubation was in MBS alone - tracks 7 and 9 show oocytes with no attached follicle cells; tracks 8 and 10, oocytes with some cells remaining. Tracks 11 and 12: oocytes radioactively labelled in the presence of $1\mu\text{g/ml}$ Hoechst 33258. Tracks 13 and 14: oocytes incubated in 0.25% collagenase in Barth's A solution for 2h before labelling. Tracks 15 and 16: oocytes incubated in Barth's A solution for 2h before labelling. Actin is indicated A; hsp70, 70.

incubated overnight in MBS containing 0.25% sheep hyaluronidase. It was anticipated that, by breaking down hyaluronic acid in the vitelline layer, this enzyme might promote the loss of follicle cells. After staining with Hoescht dye, the oocytes were divided into groups according to the approximate number of cells adhering to them and radioactively labelled at either 20°C or 35°C as usual.

The results of this experiment are shown in figure III.5. Various controls were also carried out, to check that none of the treatments imposed on the oocytes affected the synthesis of any protein, and of hsp70 in particular. These were: firstly, to label some oocytes in the presence of the Hoescht dye (tracks 11 and 12); secondly, to incubate in collagenase for two hours without removing the theca, then incubate in MBS overnight (tracks 13 and 14); and finally, since the collagenase treatments were done in Barths' A solution (which is calcium-free), to incubate in this solution for two hours then proceed as before (tracks 15 and 16). None of these treatments adversely affected protein synthesis in folliculated oocytes.

Tracks 7 and 9 show the results for oocytes which had lost all their follicle cells during incubation in MBS overnight after removal of the theca. In the non-heat-shocked sample, the overall level of protein synthesis was not lower than that in the untreated controls (track 1), illustrating one of the types of variation discussed in section III.D.1. In the heat-shocked oocytes, the level of synthesis was reduced compared with the controls, and, as expected, no detectable hsp70 was synthesized. The samples in tracks 8 and 10, however, contained oocytes which, although they had lost most

of their follicle cells, still had some sheets of cells attached. It is clear that these remaining cells were able to synthesize protein and, since hsp70 is clearly visible in track 10, were able to mount a heat shock response.

The results for oocytes incubated overnight in hyaluronidase were similar (tracks 3 to 6). Although the exact numbers of follicle cells remaining were not determined, it seemed that this treatment had promoted the release of cells from the vitelline layer, since only a few isolated clumps were found. Even so, these synthesized protein at a significant rate, and hsp70 was induced by a heat shock (track 6).

As it has already been demonstrated (III.D.iii) that follicle cells do not influence the heat shock response in oocytes, the hsp70 seen in tracks 6 and 10 must be made in the remaining follicle cells. It is important to note that relatively small isolated sheets of follicle cells can survive and respond to increased temperature normally, and that the hsp70 synthesized can readily be detected on one-dimensional gels. Clearly, experiments conducted using an inefficient method of defolliculation would lead to conclusions about the nature of the heat shock response of Xenopus oocytes very different from those we have drawn from our results.

III.F Unusual type of heat shock response

In about 30% of oocyte batches, the pattern of protein synthesis was completely different from that described above. This abnormal response is illustrated dramatically in figure III.6a, which shows the pattern observed in a batch of oocytes which actually increased their rate of protein synthesis on heat shock. With this type of response, several unusual proteins were found in the oocyte itself. These were generally present at 20°C (track 2), but were further induced by heat shock (track 4). The major bands were due to proteins of about 60, 74 and 80kD, but there was also a band of mobility indistinguishable from hsp70 on one-dimensional gels. Figure III.6, panels b-e show two-dimensional gels of these samples. The profile of heat shocked, defolliculated oocytes (panel e) is similar to that observed by Guedon et al (1985) in their study of the induction of the heat shock response in folliculated oocytes (see I.B.v), and by Browder et al (1987) in their study of developmental changes in the heat shock response.

The 60kD band in figure III.6a was due to several proteins of that molecular weight, marked 3 in panel e; protein 4 was responsible for the 74kD band, and proteins 5 and 6 both contributed to the 70kD band. Interestingly, the proteins labelled 1 and 2 in figures III.3 and 4 were less prominent in this experiment, particularly during heat shock. Panel f shows a mixture of a sample of heat shocked defolliculated oocytes and a sample containing follicle-cell-derived hsp70. Neither of proteins 5 and 6 comigrated with hona_fide hsp70 (marked H), so the 70kD protein induced in oocytes in this more unusual type of heat shock response was

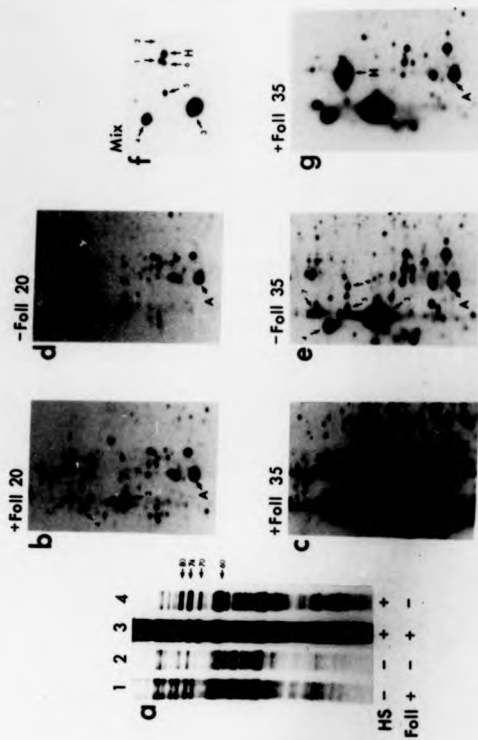


FIG. III.6 UNUSUAL PATTERN OF PROTEIN SYNTHESIS.

Oocytes were incubated for 2h in PBS containing $1\mu\text{Ci/ml}$ [^{35}S]methionine, then analysed by one- and two-dimensional electrophoresis: track 1 and panel (b), folliculated oocytes labelled at 20°C ; 2 and (d), oocytes labelled at 20°C then defolliculated; 3 and (c) [and, in a lower autoradiographic exposure, (g)], oocytes labelled at 35°C ; 4 and (e), oocytes labelled at 35°C then defolliculated; (f), a mixture of a sample of heat shocked defolliculated oocytes that exhibited this unusual response with a sample containing follicle cell-derived hsp70. Actin is indicated A; hsp70, H; proteins 1-6 are discussed in III.F.

not hsp70. The follicle cells did, however, synthesize bona fide hsp70, probably in addition to the abnormal proteins, since it was present in the sample of folliculated oocytes (panel c and, in a lower exposure, panel g).

I cannot explain the prominence of these unusual 60, 70, 74 and 80kD proteins in some batches of oocytes and not in others. One possibility is that they are modified forms of bona fide hsp70 (for example the 70kD proteins might be phosphorylated). This seems unlikely, however, since no intermediates or unmodified hsp70 were ever seen. So the most probable explanation is that they are entirely different proteins. Their expression could be due to a polymorphism in some aspect of the control of the heat shock response, or, alternatively, to the imposition of some unidentified form of stress on the frog or the oocytes before the experiments were carried out. There is circumstantial evidence that the latter possibility is nearer the truth, since in one experiment where oocytes from the same frog were used on successive days, those used one day after ovariectomy gave the normal response, whereas those used after two days gave the abnormal response (data not shown).

This conclusion is apparently supported by the data of Browder et al (1987). They routinely observed this unusual response, but found that it was dependent on the addition of pyruvate and oxaloacetate to the medium. This implies that environmental factors can indeed modulate the response of oocytes and follicle cells to heat shock. However, on the one occasion when I incubated folliculated oocytes overnight in MBS supplemented with 1mM oxaloacetate and 1.15mM pyruvate before radioactive labelling, they mounted a normal heat shock

response in exactly the same fashion as oocytes incubated in standard MBS (data not shown). So it seems that pyruvate and oxaloacetate do not invariably induce the unusual response. Unless they are actually detrimental to oocytes, it does seem odd that supplementation of the medium produced this type of response in the experiments of Browder et al. since in our experiments it tended to occur in oocytes maintained for two days in MBS, which were presumably in less good condition than immediately after ovariectomy.

The presence of these proteins does not alter the conclusion that hsp70 synthesis can never be detected in oocytes, and emphasizes the need for two dimensional analysis to distinguish bona fide hsp70 from proteins of similar molecular weights. Moreover, it suggests that in certain circumstances at least, oocytes are capable of mounting a form of heat shock response, as defined by the preferential translation of some mRNAs.

III.G Discussion

The careful examination of protein synthesis in oocytes and follicle cells described in this chapter demonstrates that the hsp70 seen when folliculated oocytes were subjected to a heat shock was actually synthesized entirely by the follicle cells. Even when oocytes themselves apparently synthesized some hsp's, none was the same as the major follicle cell hsp70. Follicle cells appear to mount the 'classical' somatic heat shock response: on heat shock they transcribe large amounts of hsp70 mRNA, which is preferentially translated, and which is degraded on return to normal temperatures.

The results of Bienz and Gurdon (1982), can be entirely explained by these findings. What appeared to be repeated masking and unmasking of hsp70 mRNA in the oocytes was actually the repeated induction and repression of the heat shock response of the follicle cells. Bienz and Gurdon did analyse proteins synthesized by defolliculated oocytes, and find that hsp70 was induced by heat shock. However, these results are misleading since in their experiments the untreated oocytes were placed directly in hypertonic medium, and both layers of cells removed in one operation. This method does not reliably remove all adhering cells (Ford and Gurdon, 1977).

A further important advantage of the method described here (III.B) is that the absence of follicle cells can be confirmed on the oocytes analysed themselves, whereas, with other methods, a parallel sample of oocytes must be fixed and sectioned. It is conceivable that follicle cells might remain attached during radioactive labelling, but fall off during the fixation of parallel samples. The experiment described in III.E suggests that any isolated cells remaining during labelling would have been capable of mounting a normal heat shock response.

One of the results reported by Bienz and Gurdon is slightly puzzling, though: they found that hsp70 was synthesized even when oocytes and follicle cells were incubated in α -amanitin, which should inhibit transcription. The probable explanation is that the follicle cells are impermeable to α -amanitin. Indeed, King and Davis (1987) found that incubation in actinomycin D (another inhibitor of transcription) prevented hsp70 synthesis completely.

Despite this, our results are not entirely compatible with the conclusions drawn by King and Davis (1987) either. Because, like us they could find no evidence that hsp70 was synthesized in oocytes, they concluded that oocytes contained no hsp70 mRNA and had no heat shock response. But we have demonstrated that oocytes do indeed contain hsp70 mRNA, so it is possible that they might synthesize heat shock protein. The levels of RNA are low, though - around 1 to 3 pg per oocyte, or 100 times less than is made by an oocyte's complement of follicle cells during heat shock - so it is possible that the translation products, if any, would be below the limits of detection of the experiments I have described. In an effort to resolve this problem, I examined oocyte polysomes for the presence or absence of hsp70 mRNA. Unfortunately, these experiments were inconclusive, because of the very small amount of the RNA present. The question of translational control of endogenous hsp70 message therefore remains unresolved.

CHAPTER IV: RESULTS AND DISCUSSION

TRANSCRIPTION OF hsp70 GENES IN XENOPUS OOCYTES

IV.A Introduction

The results described in Chapter III do not resolve the question of the translational status of the hsp70 mRNA in *Xenopus* oocytes. No hsp70 synthesis could be detected, but since hsp70 mRNA was present in small amounts, it remained a possibility that this was translated under some circumstances. To test whether oocytes were capable of making hsp70 if they were provided with more hsp70 RNA, they were injected with hsp70 DNA, and protein synthesis was followed in oocytes incubated at normal and heat shock temperatures.

In these initial experiments, hsp70 DNA, rather than RNA synthesized in vitro, was injected into oocytes since it could be argued that RNA transcribed in vivo was more likely to be regulated appropriately. It was already known that the *Xenopus* hsp70 promoter is active at 20°C in oocytes (Bienz, 1984b). However, for the results of protein labelling experiments to be readily interpretable, it was necessary for the same amount of transcript to be present in the cytoplasm both before and after a heat shock, and for the newly transcribed RNA to be translatable. This chapter describes the DNA constructs injected in our experiments, their transcription by oocytes, and the nature and location of the resulting transcripts.

IV.B hsp70 genes

Genomic clones of two *Xenopus* hsp70 genes have been isolated by Bienz (1984b). One (hsp70A) has been completely sequenced, and the other (hsp70B) has been partially sequenced (Biernz 1984b). Additional information on the hsp70B gene has been obtained in this laboratory by Dr John Shuttleworth (fig. IV.1a). In addition to the single base changes already reported at positions -13, -1, 18, 21 and 46, the hsp70B leader contains an insertion of seven bases at positions 102 to 108.

The promoters and entire coding sequences of the hsp70A and hsp70B genes have been subcloned into pUC12. These constructs were named pXL10XP and pXL16P respectively by Bienz (1984b), but for simplicity I refer to them as phsp70A and phsp70B. Although the promoters of both genes, and the coding sequence of hsp70A, have been sequenced, the constructs also contain lengths of DNA 5' and 3' to the gene which have not been sequenced. Some of the restriction sites in these regions became apparent while I was carrying out manipulations on the hsp70 genes, and the approximate positions of these sites are marked in figure IV.1b.

For the RNase protection assay used to analyse oocyte RNA, the AhaIII to HindIII fragment was inserted into pGEM-2 and a 506-nucleotide antisense probe synthesized in vitro using SP6 RNA polymerase (see II.J.iv). In the assay, hsp70B RNA protected 476 nucleotides, corresponding to the entire probe apart from 17 nucleotides of vector sequence and the 13 nucleotides from the AhaIII site to the cap site. In contrast, the fragment protected by hsp70A RNA was only 368 nucleotides because of the mismatch between it and the hsp70B probe

```

70B -16  AhaIII      {-----B
          TTGAAGCGATGTGGTGGTAGCCGCGATCTAAGCAGATACTTGTGAGATCTGACAGAGAAG
70A -16  TTGAAGCGATGTGGAGGTAGCAGAACTCAAGCTGACAC1TGTGAGATCTGACAGAGAAG
          |
          Csp
          *
45  AACAACTGCACAGCGATACTTACTGGGCAAGAGCGAGCTGCNGATATCTAGCGAAAAG
45  AGCAACTGCACAGCGATACTTACTGGGCAAGAGCGAGCTGCNGATATCTAGCGAAA
          *
          ****
105  CGTGAAGCAAACTGTAGGAGCSCAAAT@TGCACCAAAAGAGATCGCAGTCGGCATTGA
102  AAGCAAACTGTAGGAGCSCAAAT@TGCACCAAAAGAGATCGCAGTCGGCATTGA
          {-----A

165  CCTCGGCACTACCTACTCTTGCSTTGGTGTTCAGCATGGTAAAGTAGAGATCATCGC
158  CCTCGGCACTACCTACTCTTGCSTTGGTGTTCAGCATGGTAAAGTAGAGATCATCGC

225  TAATGACCAAGGGAATCGCACCACCTCCGAGTATGTGGCTTCACAGACACAGAAAGCT
218  TAATGACCAAGGGAATCGCACCACCTCCGAGTATGTGGCTTCACAGACACAGAAAGCT

285  GATTGGCGATGCAGCAAGAATCAAGTGGCCATGAACCCCGAATACAGTGTTTGATGC
278  GATTGGCGATGCAGCAAGAATCAAGTGGCCATGAACCCCGAATACAGTGTTTGATGC

345  CAAGAGACTGATCGGAGAAAGTTCAATGATCTGTAGTGCAGTGTGACTTGAAGCACTG
338  CAAGAGACTGATCGGAGAAAGTTCAATGATCTGTAGTGCAGTGTGACTTGAAGCACTG

405  GCCGTTCGAAGTGGTAGCGATGAGGGGAAGCCTAAGGTCAAAGTGGAGTATAAAGGAGA
398  GCCGTTCGAAGTGGTAGCGATGAGGGGAAGCCTAAGGTCAAAGTGGAGTATAAAGGAGA

          B-----}
465  AGAGAAAGCTT
458  AGAGAAAGCTT
          A-----}

```

FIG. IV.1A. THE 5' SEQUENCE OF GENOMIC hsp70A And hsp70B CLONES

The 484bp AhaIII-HindIII fragment of phsp70B was inserted into M13mp18 and mp19 and sequenced by dideoxynucleotide chain termination. Its sequence is compared with hsp70A (Brenz, 1984b); mismatches are indicated (*); the cap sites and initiator ATG codons are underlined; the 5' and 3' limits of probe protection in the RNase assay are indicated (<--A-->, <--B-->).

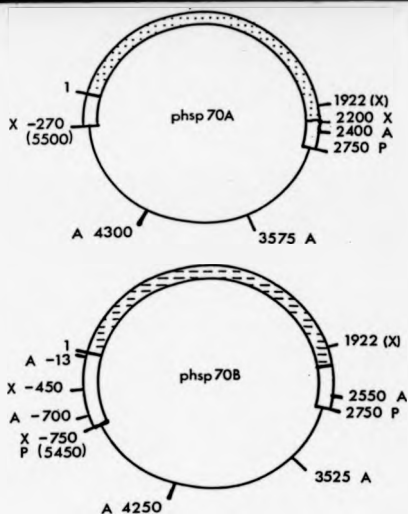


FIG. IV.1b. RESTRICTION SITES IN phsp70A AND phsp70B.

The hsp70A and hsp70B genes were subcloned in pUC12 (Blenz, 1984b). The position of all sites indicated is approximate, except for those within the promoters and coding regions of the genes. All restriction sites in the coding regions were as predicted from the complete hsp70A and partial hsp70B sequences (Blenz, 1984b); the regions upstream of -260 and downstream of the polyadenylation site have not been sequenced, and the restriction enzyme sites differ greatly between the hsp70A and B genes. Stippled box, hsp70A transcribed sequences; shaded box, hsp70B transcribed sequences; open boxes, DNA flanking hsp70 genes; black lines, pUC12 sequences. A, AhaIII sites; X, XbaI sites; P, PstI sites.

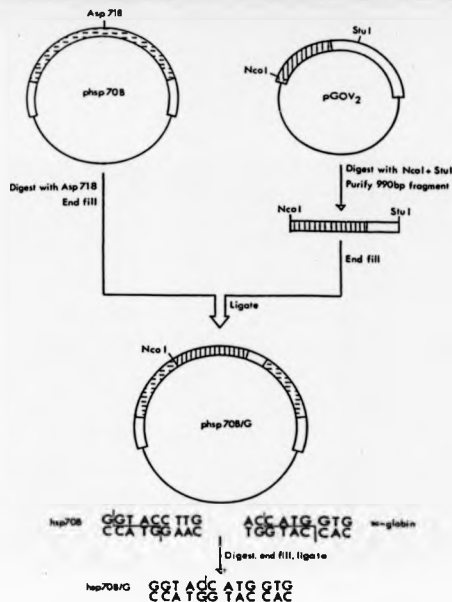


FIG. IV.2 CONSTRUCTION OF pshp70B/G.

After digestion of pGOV₂ with NcoI and StuI, the 990bp fragment was purified from an agarose gel, and made blunt ended by end filling using Klenow enzyme (II.B and II.C). This was ligated with pshp70B which had been linearized with Asp718, made blunt ended in the same way, then treated with CIP. Shaded box, hsp70B transcribed sequences; vertically hatched box, α-globin; open boxes, flanking sequences (in pshp70B) and ovalbumin sequences (in pGOV₂).

The sequence of the fusion point is also shown. The fusion is in frame, and no new codons are created.

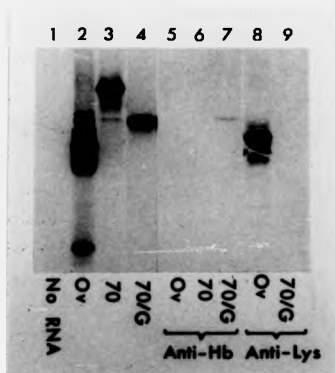


FIG. IV.3 IN VITRO TRANSLATION OF hsp70B/A AND hsp70B/B SP6 RNA.

Reticulocyte lysate translations (II.K) were carried out with the following additions: track 1, no RNA; track 2, 100ng oviduct poly(A)+ RNA; track 3, 100ng hsp70B/A SP6 RNA; track 4, 100ng hsp70B/B. One tenth of each translation mix (i.e. 1 μ l) was loaded per track. Before analysis by SDS-PAGE, equivalent volumes of certain samples were immunoprecipitated using rabbit anti-haemoglobin: track 5, oviduct; track 6, hsp70B/A; track 7, hsp70B/B. Some samples were also immunoprecipitated with rabbit anti-lysozyme: track 8, oviduct; track 9, hsp70B/B.

RNA.

To check that the join between the hsp70B and globin genes was indeed in frame, the fragment between the AhaIII site at position -13 in hsp70B and the PstI site at the 3' end of the globin gene was subcloned into pGEM-2 (see fig. VI.1b). Capped RNA was transcribed by SP6 RNA polymerase from this construct in vitro, then translated in reticulocyte lysate. The resulting radiolabelled protein was immunoprecipitated and analysed on an SDS-polyacrylamide gel (fig. IV.3). This confirms that the product of the fusion gene was, as predicted, a polypeptide of about 50kD (35kD of hsp70 and 15kD of globin, track 4). Track 3 shows the translation product of an SP6 RNA coding for native hsp70 (actually a fusion of the hsp70A and hsp70B coding sequences; see fig. VI.1a). As expected, the fusion protein could be precipitated with the anti-haemoglobin antibody (track 7), while hsp70 and ovalbumin could not (tracks 5 and 6), and it could not be precipitated with anti-lysozyme (track 9), while ovalbumin could (track 8). However, the fusion protein was precipitated very inefficiently (compare tracks 4 and 7, which represent equal volumes of translation mix). This was probably because the conformation of globin was altered by being fused to hsp70.

IV.D Transcription of injected hsp70A and hsp70B genes in oocytes

The experiments described in this section were all carried out by Dr John Shuttleworth.

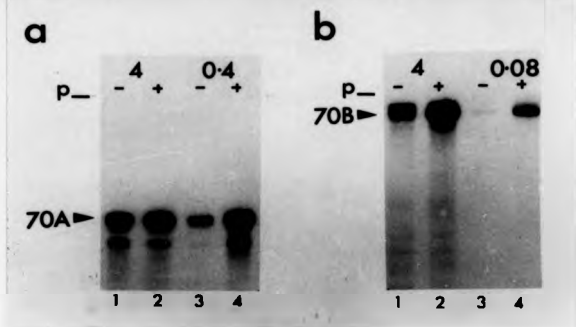


FIG. IV.4 TRANSCRIPTION FROM INJECTED hsp70 GENES. After injection of phsp70A or phsp70B DNA, oocytes were incubated at 20°C for 24h. Total RNA was extracted from oocytes, which were defolliculated immediately before homogenization, either before (-) or after (+) a 2h heat shock at 35°C, and analysed by the RNase protection assay.

Panel a: phsp70A injected at 4ng (tracks 1 and 2) or 0.4ng (tracks 3 and 4) per oocyte.

Panel b: phsp70B injected at 4ng (tracks 1 and 2) or 0.1ng (tracks 3 and 4) per oocyte.

Probe protected by hsp70A transcripts, 70A; probe protected by hsp70B transcripts, 70B; residual full-length probe, p. The fragment migrating ahead of the hsp70A-protected fragment does not occur reproducibly and is either an artifact of the assay or produced from degraded hsp70A transcripts. The fragment migrating slower than the hsp70B transcript corresponds to hsp70 transcripts initiating upstream of the normal cap site. A shorter exposure of the gel revealed that these transcripts did not increase on heat shock.

RNA equivalent to one oocyte was analysed in each case.

IV.D.i Rate of transcription

Oocytes were injected with various concentrations of hsp70 DNA, since this, along with other factors has been reported to affect expression (Biens, 1988). They were incubated at 20°C for 24 hours, then hsp70 transcripts were assayed both before and after a two hour heat shock. Representative results are illustrated in figure IV.4. Both hsp70A and hsp70B genes were transcribed at 20°C, although hsp70A was transcribed more efficiently: when injected with 4ng of hsp70A DNA, oocytes accumulated 3.4ng hsp70A RNA within 24 hours (fig. IV.4a, track 1), whereas they accumulated only 1ng of hsp70B RNA after injection of the same amount of hsp70B DNA (fig. IV.4b, track 1). When lower amounts of DNA were injected less RNA was synthesised, although the effect was not proportional. These results are in agreement with those of Biens (1984b and 1988).

When the oocytes were given a two hour heat shock, however, there was a detectable increase in hsp70 transcripts in all cases (fig. IV.4a and b). This effect was most dramatic when oocytes were injected with the lower amounts of DNA (fig. IV.4a, tracks 3 and 4; fig. IV.4b, tracks 3 and 4). In this experiment, for oocytes injected with 0.4ng of hsp70A, the induction of transcription on heat shock corresponded to a rate of 21 transcripts per gene per hour (or an additional 3.75ng of RNA), while for those injected with 4ng of DNA the rate was only 0.05 transcripts per gene per hour (an additional 100pg). The extent of induction of hsp70A transcription also varied considerably between experiments.

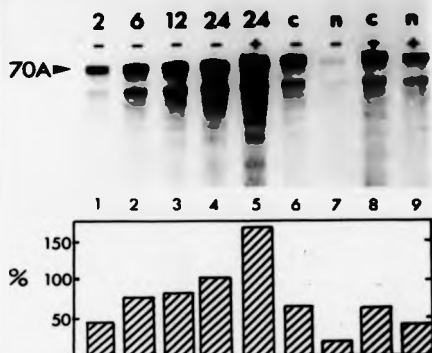


FIG. IV.5 ACCUMULATION AND LOCATION OF RNA TRANSCRIBED FROM INJECTED hsp70A GENES. Oocytes were injected with 4ng of hsp70A DNA per oocyte. After defolliculation of the oocytes, total RNA was extracted after 2, 4, 12 or 24h at 20°C (tracks 1, 2, 3 and 4 respectively) or after 24h at 20°C followed by 2h at 35°C (track 5). RNA was also extracted from the cytoplasm (c) and nuclei (n; see II.N.ii) of oocytes incubated at 20°C for 24h (tracks 6 and 7) or at 20°C for 24h followed by a 2h heat shock at 35°C (tracks 8 and 9). RNA equivalent to one oocyte was analysed by RNase protection in each case. The position of probe protected by hsp70A transcripts is indicated by an arrow. The lower panel indicates the relative amounts of hsp70A transcripts in each sample, assessed by determining the radioactivity in bands corresponding to protected fragment excised from the gel. The level of transcripts accumulated after 24h at 20°C (track 4) was taken as 100%.

By contrast, the results with all concentrations of hsp70B showed a significant increase in the level of hsp70 transcripts following a heat shock. This difference between the behaviour of the hsp70A and hsp70B genes is puzzling, because the sequences of their promoter regions (at least as far upstream as -382) are almost identical. It is possible that it is due to signals further upstream, however, since the hsp70B plasmid contains approximately 750 base pairs upstream of the cap site, whereas the hsp70A plasmid contains only 252 base pairs.

IV.D.ii Location of the transcripts

The finding that heat shock does in fact cause an increase in the level of hsp70 transcripts in injected oocytes would, by itself, make interpretation of translation data very difficult, since a change in rate of synthesis might be a reflection of changes in RNA levels, rather than rates of translation. However, an early experiment with X. borealis oocytes indicated that the heat shock induced transcripts were predominantly in the nucleus and therefore unavailable for translation.

This experiment was repeated in more detail using X. laevis oocytes (fig. IV.5) into each of which had been injected 4ng hsp70A DNA and which were separated into cytoplasm and nucleus before analysis. At 20°C, 80% of the hsp70 transcripts were in the cytoplasm (tracks 6 and 7). A two hour heat shock induced a 68% increase in hsp70A transcripts in this experiment, but all these new transcripts were indeed retained in the nucleus. The number of transcripts available for translation therefore remained constant, despite

the induction of transcription.

The results in figure IV.5 also show the accumulation of transcripts during the 24 hour incubation at 20°C (tracks 1-4). They suggest that rather than being constitutively active in oocytes (Bienz 1984b and 1986), hsp70 genes might only be active for the first few hours after injection. The modest increases in transcript levels seen on heat shock 24 hours after injection might therefore reflect a dramatic induction of transcription of hsp70 genes that are by then quiescent.

The distribution of hsp70B transcripts in injected oocytes was similar, except that even at 20°C a significant proportion remained in the nucleus (data not shown). Coupled with the lower rate of transcription at 20°C and the greater induction seen on heat shock than was found with hsp70A, this means that little hsp70B RNA was present in the cytoplasm at 20°C, and that this amount did not increase on heat shock. The behaviour of hsp70/G genes and transcripts in oocytes was the same as for hsp70B, as would be expected, since the promoter region of hsp70B was unaltered during the construction of the fusion gene.

IV.1 Sizes of the transcripts from injected genes

Transcription of eukaryotic genes continues past the 3' end of the mature mRNA, sometimes by as much as several hundred nucleotides. Subsequent processing by cleavage and polyadenylation requires the presence of specific sequences 10-30 nucleotides upstream and, at least in some genes, 15-50 nucleotides downstream of the polyadenylation site (Gil and Proudfoot, 1987; see also Birnstiel *et al.* 1985 for review).



FIG. 1V.6a NORTHERN BLOT SHOWING hsp70 TRANSCRIPTS IN FOLLICLE CELLS AND INJECTED OOCYTES. Total RNA was extracted from oocytes either before (-) or after (+) a 2h heat shock, and analysed by northern blot ([I.J.iii]), using the 50A-nucleotide hsp70B probe. RNA equivalent to one oocyte was loaded onto each track. Tracks 1, 2, and 7, folliculated oocytes; 3 and 4, oocytes injected with 4ng phsp70A, incubated overnight at 20°C and defolliculated immediately before homogenization; 5 and 6, oocytes injected with 10ng phsp70B, incubated at 20°C overnight and defolliculated before homogenization.

FIG. 1V.6b NORTHERN BLOT ANALYSIS OF *X. borealis* OOCYTE RNA. Total RNA was extracted from *X. borealis* oocytes and analysed as described above. Track 2, oocytes injected with 0.1ng phsp70A, incubated overnight at 20°C and defolliculated; 3, oocytes injected with 4ng phsp70B, incubated overnight, and defolliculated; 4 and 5, oocytes injected with 4ng phsp70B/B, incubated overnight and defolliculated; 6, 7 and 8 (which is a longer exposure of track 7), folliculated, uninjected oocytes; 1, folliculated *X. laevis* oocytes.

B, hsp70B/B transcripts; N, hsp70 transcripts; C, 'hsp70 cognate' transcripts.

Since the hsp70A and hsp70B plasmids injected both contain approximately 500bp of genomic sequence downstream of the polyadenylation site, it was anticipated that the sequences necessary for transcript processing would be present. However, the RNase mapping data discussed above do not show whether transcripts are processed correctly or indeed whether they are full length. Northern blots were therefore, carried out on RNA extracted from injected oocytes to determine the length of hsp70 RNA molecules transcribed from the injected genes. The results of two such blots are shown in figure IV.6. SP6 transcripts of known length were used for size estimation (data not shown).

Figure IV.6a shows the results of a northern blot of RNA from *X. laevis* oocytes injected with hsp70A and hsp70B DNA, and from control oocytes complete with their follicle cells. A band corresponding to transcripts of approximately 2.4kb is visible in all tracks, including non-heat-shocked controls (track 1). This is presumably due to hybridization to mRNA coding for heat shock cognates (Craig *et al.* 1983). A heat shock induced the appearance of a transcript of about 2.8kb in control oocytes, which was due to the induction of follicle cell hsp70 mRNA synthesis (track 2). Apart from the 2.4kb transcript, there was one major product in oocytes injected with hsp70A DNA (tracks 3 and 4). This comigrated with the heat-inducible band in track 2. Transcripts both longer and shorter than this were also detected, but they were always minor products, and were present in uninjected oocytes as well. The heat inducibility of hsp70B genes is illustrated dramatically by tracks 5 and 6: a band corresponding to RNA transcribed from injected hsp70B is visible only in the RNA

from heat shocked oocytes.

The transcripts from the hsp70B/globin fusion gene are illustrated in figure IV.6b, which shows the results of injecting heat shock genes into X. borealis. The major transcripts from the hsp70A and B genes were again indistinguishable from those induced in X. laevis by heat shock (tracks 1-3). However, RNA transcribed from the fusion gene (phsp70B/G) was estimated to be at least 500 nucleotides longer (tracks 4 and 5). This suggests that transcription did terminate at the end of the hsp70B gene as predicted (see IV.C), rather than in the globin gene inserted into it.

It is interesting to note that very little cross-hybridization was observed between the X. laevis hsp70B probe and the X. borealis follicle cell hsp70 mRNA. Even on overexposure of the autoradiograph the band corresponding to X. borealis follicle cell hsp70 mRNA was very faint (track 8). This effect was also observed in RNase mapping assays of X. borealis RNA, and indicates either that there is surprisingly little homology between the 5' ends of heat shock genes of the two species, or that no induction of hsp70 gene transcription occurs in X. borealis follicle cells.

IV.F Polyadenylation status of transcripts

Most natural mRNA species do, of course, have a poly(A) tail at their 3' end, and work in this laboratory has indicated this increases both the stability and the translatability of the RNA in oocytes (Drummond et al. 1985). If the RNA transcribed from hsp70 genes injected into oocytes was to be translatable, it was obviously important, therefore, that it was polyadenylated. Its polyadenylation status was

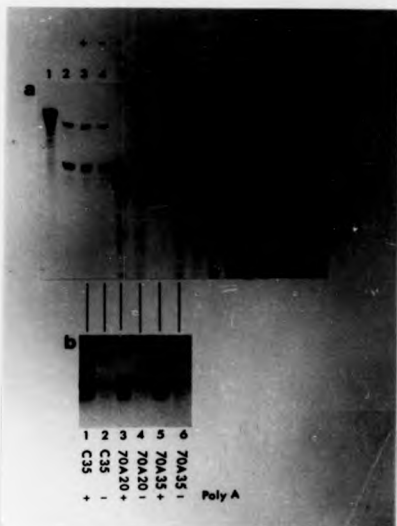


FIG. IV.7a POLY(A) STATUS OF hsp70 TRANSCRIPTS.

Oocytes were injected with 4ng phsp70A (tracks 5-8), phsp70B (tracks 9-12) or phsp70B/G (tracks 15 and 16), and incubated overnight at 20°C. RNA was extracted from oocytes (defolliculated immediately before homogenization) either before or after a 2h heat shock at 35°C, and poly(A)+ RNA was selected by binding to oligo-dT cellulose (II.J.ii). The poly(A)+ and poly(A)- fractions were analysed by RNase protection. Tracks 5 and 6, phsp70A -HS; 7 and 8, phsp70A +HS; 9 and 10, phsp70B -HS; 11 and 12, hsp70B +HS; 15 and 16, phsp70B/G -HS. RNA was also extracted from heat shocked folliculated oocytes and analysed in the RNase protection assays; track 2, total RNA; 3, poly(A)+; 4, poly(A)-. RNA equivalent to one oocyte was analysed in each case. Track 1, full-length probe; 13, pBR322/HpaII markers (622, 527, 404, 309, 242, and 238 bp); 14, 10µg tRNA RNase protection assay control.

FIG. IV.7b NORTHERN BLOT OF OVIDUCT POLY(A)+ RNA SELECTED BY OLIGO-dT CELLULOSE.

Oviduct poly(A)+ (5ng per oocyte equivalent of total RNA) was added to total RNA before selection by oligo-dT cellulose. Oocyte poly(A)+ and poly(A)- fractions were then analysed by northern blot, using a nick-translated ovalbumin gene fragment as a probe. Tracks 1 and 2, uninjected folliculated oocytes, poly(A)+ and poly(A)-; 3 and 4, phsp70A injected, non-heat shocked oocytes, poly(A)+ and poly(A)-; 5 and 6, phsp70A-injected, heat shocked oocytes, poly(A)+ and poly(A)-. RNA equivalent to one oocyte was analysed in each case.

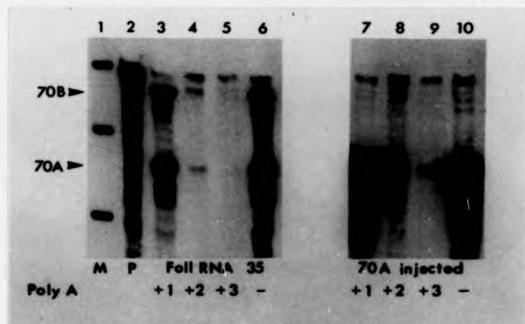


FIG. IV.B REPEATED OLIGO-dT SELECTION OF hsp70 RNA.

Total RNA was extracted from heat shocked *X. laevis* oocytes plus follicle cells (tracks 3-6), and from *X. borealis* oocytes each injected with 10ng hsp70A DNA, incubated overnight at 20°C then heat shocked for 2h before being defolliculated and homogenized. Poly(A)⁺ RNA was selected with oligo-dT cellulose (see II.J.i) (tracks 3 and 7). The 'poly(A)⁻' fractions were then subjected to two more rounds of oligo-dT selection (4, 5, 8, and 9). Tracks 6 and 10 therefore show RNA which failed to bind to oligo-dT cellulose during three rounds of selection. All fractions (one oocyte equivalent in each case) were analysed by RNase protection (II.J.iv). Track 1, radioactive pBR322/HpaII markers (527, 404, and 309bp); track 2, full-length probe.

assessed by its ability to bind to oligo-dT cellulose. RNA in the resulting fractions, designated 'poly(A)+' and 'poly(A)-', was assayed by RNase mapping. The results are shown in figure IV.7a. For all samples tested, including RNA from heat shocked follicle cells, approximately equal counts were eluted from the gel bands corresponding to both '(A)+' and '(A)-' fractions, although a higher proportion of the RNA induced on heat shock was 'poly(A)-'.

The presence of RNA in the '(A)-' fraction could have been due to lack of a poly(A) tail long enough for it to bind to oligo-dT cellulose (i.e. 20-30 nucleotides; see Rosenthal and Ruderman, 1987), or to failure of RNA with longer poly(A) tails to bind to the oligo-dT cellulose for some experimental reason. However, the latter was discounted by subjecting the RNA to analysis on a northern blot. The probe used was nick-translated ovalbumin DNA, which hybridized to oviduct poly(A)+ RNA which had been added to the oocyte RNA before selection by oligo-dT. This RNA was almost entirely confined to the '(A)+' fraction, indicating that the selection process had been working well (figure IV.7b).

This conclusion was confirmed by conducting an experiment in which RNA from both heat shocked *X. laevis* follicle cells (fig. IV.8 tracks 3-6) and *X. borealis* oocytes injected with hsp70A DNA (tracks 7-10) was subjected to several rounds of oligo-dT selection. Relative amounts of RNA in the different fractions were estimated by comparing cpm eluted when the corresponding bands were cut out of the gel. Since hybridization of the probe to sense hsp70 RNA was found to be linear over the range of 0-5000 pg (Horrell *et al.* 1987), this procedure would have given reasonably accurate

estimates. In both cases, about 55% of the hsp70 RNA remained in the '(A)-' fraction after three rounds of selection with oligo-dT cellulose (tracks 6 and 10). Since the amounts of RNA in the second '(A)+' fraction were only about 10% of those in the first, and negligible amounts were found in the third fractions, saturation of oligo-dT with poly(A)+ RNA was obviously not a problem. It appears, therefore, that on a certain proportion of endogenous hsp70 mRNA the poly(A) tail is either too short to allow binding to oligo-dT, or absent altogether.

IV.G Discussion

The results described in this chapter are surprising, because previously published data had indicated that hsp70 genes are not heat inducible in *Xenopus* oocytes (Bienz, 1984b and 1986). The differences between our results and those of Bienz may be due to the fact that she analysed transcript levels only two hours after injection, whereas we incubated oocytes for 24 hours at normal temperatures before analysis, so that large amounts of the transcript accumulated. The time-course of hsp70 RNA accumulation (fig. IV.5) indicates that two hours after injection the genes are actively transcribed even in the absence of heat shock, and it is possible that the induction on heat shock is insignificant against this background. By contrast, after 24 hours the genes are transcribed very slowly at 20°C, and the induction of transcription by heat shock is more dramatic.

Our findings none the less allowed the rational design of experiments to test the ability of oocytes to translate hsp70 mRNA. Injected hsp70A genes were efficiently transcribed

at 20°C, thus increasing the amount of hsp70 RNA in the cytoplasm by up to 1000-fold. Although transcription was induced further by a heat shock, the resulting RNA was not exported to the cytoplasm, so that the amount of RNA available for translation remained constant throughout a two hour heat shock. Moreover, the length and polyadenylation state of this RNA were indistinguishable from those of hsp70 mRNA induced in follicle cells by heat shock, so it was expected to be translatable. It is interesting that, in both populations, the proportion of poly(A)- RNA was rather high (about 50%).

However, it seems that the choice of the hsp70B gene for construction of a fusion gene was unfortunate: before heat shock, less RNA was transcribed from this promoter and less found in the cytoplasm than with hsp70A. Although induction of transcription on heat shock was greater, this induced RNA also remained in the nucleus. The amount of RNA in the cytoplasm was therefore always less than when hsp70A DNA was injected, suggesting that the resulting protein would be more difficult to detect.

CHAPTER V. RESULTS AND DISCUSSION

EXPRESSION OF hsp70 FROM INJECTED GENES

V.A Introduction

Since no hsp70 could be detected inside defolliculated oocytes incubated with or without a heat shock, nothing could be concluded about the translational control of the hsp70 mRNA which was found in the oocytes. In an attempt to resolve this problem, a series of experiments was conducted in which exogenous hsp70 RNA was introduced into oocytes, to determine whether they were capable of synthesizing hsp70 under any circumstances.

The main difficulty with this approach was that there were no published examples of the use of experiments of this type to investigate translational control in *Xenopus* oocytes. It was therefore not known whether exogenous RNA would be subject to the same control mechanisms as endogenous mRNA. Consequently, in the initial experiments, hsp70 DNA, rather than RNA synthesized in vitro, was injected. This was because it could be argued that RNA transcribed in the oocyte germinal vesicle, and processed and exported to the cytoplasm, was more likely to be accessible to translational control mechanisms than RNA injected in large amounts, directly into the cytoplasm.

Various other criteria were also applied to assess whether the translational control of this hsp70 RNA was specific, or merely a result of the method of its introduction into the oocytes. These included comparisons of its behaviour with, firstly, the known behaviour of hsp70 mRNA in other systems, and, secondly, the behaviour of RNA transcribed from non-heat shock genes injected into oocytes (see Chapter VII).

The results described in Chapter IV indicated both that RNA transcribed from injected hsp70 genes was indistinguishable from the follicle cell transcripts, and that, if the DNA and its concentration were chosen appropriately, a high and constant level of the transcripts was present in the cytoplasm. This would simplify interpretation of the results of protein-labelling experiments: any changes found in the synthesis of hsp70 would be due entirely to alterations in the rate of translation of pre-existing RNA; translation of newly transcribed RNA would not have to be considered.

V.B Expression of hsp70B/globin fusion protein

Several experiments were carried out in which DNA coding for the hsp70B/globin fusion gene (phsp70B/G) was injected into oocytes (see IV.C for details of construction). Each oocyte received about 4ng DNA. After incubation at 20°C for 24 hours to allow transcription to occur, the oocytes were labelled with [³⁵S]methionine for two hours at either 20° or 35°C. They were then homogenized and aliquots of each sample immunoprecipitated before being analysed on SDS-polyacrylamide gels.

In most experiments, no fusion protein could be detected, presumably for a combination of the two reasons already discussed: firstly, although transcription from the hsp70B promoter was efficient during heat shock, levels of the RNA in the cytoplasm were low both before and during a heat shock (IV.D); and secondly, the fusion protein was not efficiently immunoprecipitated by the anti-haemoglobin antibody (IV.C).

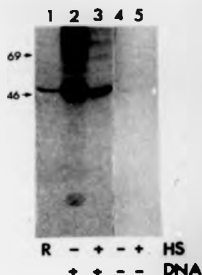


FIG. V.1 EXPRESSION OF hsp70B/8 IN OOCYTES.

Oocytes were each injected with 2ng psp70B/8 DNA, incubated overnight at 20°C, then incubated in 1mCi/ml [³⁵S]methionine for 2h at either 20°C (track 2) or 35°C (track 3). After homogenization, samples were immunoprecipitated with the rabbit anti-haemoglobin antibodies (H.K.iii), and analysed by electrophoresis on 12.5% polyacrylamide gels. Each track represents approximately 0.4 oocytes. Control immunoprecipitates of uninjected oocytes are shown in tracks 4 and 5. Track 1 shows the reticulocyte lysate translation product of the synthetic 70B/8 RNA.

Despite this, expression of the fusion protein was detected in one experiment (fig. V.1), in which oocytes mounted the normal heat shock response. Since it was present in oocytes incubated at 20°C (track 2), its RNA was obviously not masked at normal temperatures. However, it did appear to be preferentially translated during heat shock (track 3). The bands corresponding to the immunoprecipitated fusion protein were excised from the gel, and the amounts of radioactivity eluted indicated that its rate of synthesis fell by only 25% on heat shock, compared with a fall in the overall rate of protein synthesis of about 50% (estimated by acid precipitation of protein from homogenates, data not shown). This probably represents a fall of more than 50% in the rate of protein synthesis in the oocytes themselves, since follicle cells contribute a higher proportion of the overall rate during heat shock than at normal temperatures (see III.D.ii).

V.C Expression of hsp70A

Because of the difficulties encountered in inducing oocytes to synthesize detectable amounts of the fusion protein, in later experiments hsp70A DNA was injected into oocytes instead of phsp70B/G. The analysis of these experiments was more complicated, as the oocytes had to be defolliculated to distinguish protein synthesized as a result of DNA injection from that induced in follicle cells by heat shock. However, this disadvantage was greatly outweighed by the ability of oocytes to transcribe hsp70A genes efficiently and transport the RNA to the cytoplasm.

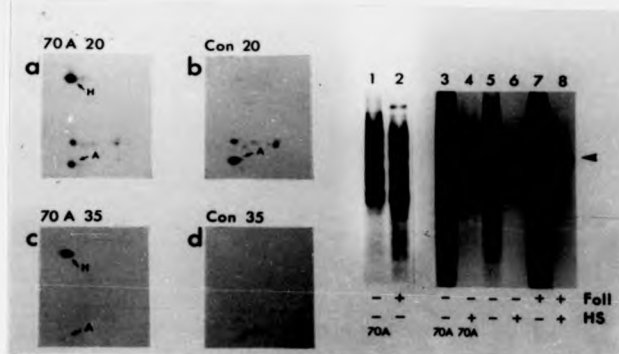


FIG. V.2 EXPRESSION OF hsp70 IN OOCYTES.

Oocytes were injected with 4ng hsp70A DNA, incubated at 20°C overnight, then incubated in [35 S]methionine for 2h at either 20°C (HS-) or 35°C (HS+). They were defolliculated, homogenized and analysed by one- and two-dimensional electrophoresis (tracks 1, 3 and 4; panels a and c). Control, uninjected oocytes were analysed on one-dimensional gels both with (Foll+) and without (Foll-) their follicle cells (tracks 2 and 5-8), and on two-dimensional gels without follicle cells (panels b and d). Tracks 1 and 2 show a shorter autoradiographic exposure of tracks 3 and 7, respectively. H, hsp70; A, actin.

In the experiment illustrated in figure V.2, about 3.4ng of hsp70A RNA was present in the cytoplasm before heat shock. On heat shock, a further 0.1ng of RNA was transcribed, but this would have been confined to the germinal vesicle (data not shown, but see IV.D.ii). After labelling with [³⁵S]methionine and subsequent defolliculation, hsp70 was readily detectable in injected oocytes incubated both at 20°C and at 35°C (fig. V.2, panels a and c). Indeed it was the major protein synthesized at both temperatures. This protein comigrated with follicle cell hsp70 on two-dimensional gels (data not shown).

In contrast, no hsp70 could be detected in control, defolliculated oocytes (panels b and d). The rate of synthesis of hsp70 was similar at the two temperatures, but the synthesis of other proteins, notably actin, was markedly inhibited during heat shock. So much hsp70 was synthesized in this experiment, that it was visible even on one dimensional gels (fig. V.2, tracks 1 and 4), although two dimensional gels were, of course, needed for unequivocal identification. [To improve clarity, a shorter exposure of the autoradiograph is shown for the samples of injected and control oocytes incubated at 20°C (tracks 1 and 2 are the same samples as 3 and 7, respectively).]

These results are similar to those obtained with the fusion protein (fig. V.1). Again the exogenous mRNA was not masked at normal temperatures, but it was selectively translated during heat shock: in other words, it was translated preferentially compared with other mRNAs.

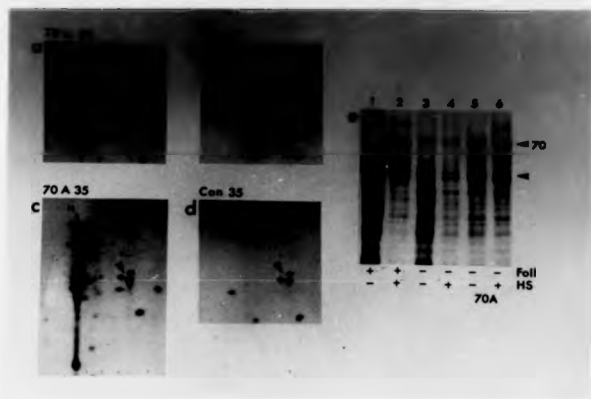


FIG. 5.3 SELF-DEGRADATION OF hsp70.

Oocytes were injected with 4ng hsp70A DNA, incubated overnight at 20°C, then incubated in [³⁵S]methionine for 2h at either 20°C (-HS) or 35°C (+HS). They were defolliculated, homogenized, and analysed on one- and two-dimensional gels (tracks 5 and 6; panels a and c). Control, uninjected oocytes were analysed on one-dimensional gels both with (+Foll) and without (-Foll) their follicle cells (tracks 1-4), and on two-dimensional gels without follicle cells (panels b and d). H, hsp70; large arrow indicates 45kD peptides.

V.D Self-degradation of hsp70

The experiment illustrated in figure V.3 gives another example of the variation in the pattern of protein synthesis between different batches of oocytes. In this case, there was a group of unusual bands corresponding to peptides of about 45kD which were present in all samples (fig. V.3a), and which were visible as a group of spots (marked with an arrow) on the two-dimensional gels. The rate of incorporation of [³⁵S]methionine into protein also varied considerably between samples incubated at the same temperature. Since equivalent amounts of total protein were loaded onto each track or tube gel, these differences are reflected in the intensities of the bands and spots on the autoradiographs.

Nonetheless, the expression of hsp70 in injected oocytes appeared to conform to the pattern described above, when analysed on one-dimensional gels (tracks 5 and 6), except that the rate of synthesis of hsp70 in injected oocytes actually increased on heat shock. However, on two-dimensional gels, the picture was very different. The spots in the region of hsp70 were unexpectedly weak (panels a and c), considering that there were clear bands due to proteins of 70kD on the one-dimensional gels. There were, though, smears of radioactivity running down the gels from that position. These appear to be due to degradation products of hsp70. Other proteins were not affected, so the effect was not due to a generalized protease activity.

The degradation must have occurred after the first dimension isoelectric focussing gel, either during equilibration or during running of the second dimension, since the degradation products would not all have the same

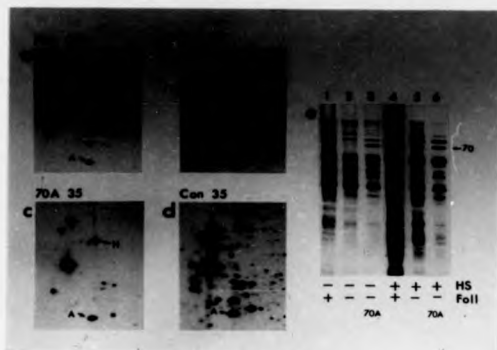


FIG. V.4 EXPRESSION OF hsp70A IN OOCYTES EXHIBITING UNUSUAL HEAT SHOCK RESPONSE.

Oocytes were injected with 4ng hsp70A DNA, incubated overnight at 20°C, then incubated in [35 S]methionine for 2h at either 20°C (-HS) or 35°C (+HS). They were defolliculated, homogenized, and analysed on one- and two-dimensional gels (tracks 3 and 6; panels a and c). Control, uninjected oocytes were analysed on one-dimensional gels both with and without their follicle cells (tracks 1, 2, 4, and 5), and on two-dimensional gels without follicle cells (panels b and d). M, hsp70; A, actin; proteins 1 and 2 are discussed in the text.

isoelectric point as hsp70 itself. This implies that the protease activity copurifies with hsp70 at least in the isoelectric focussing dimension. The most probable explanation is, therefore, that hsp70 slowly digests itself. The reason for the prominence of degradation products in this experiment may be that the gels illustrated here were run at 5mA/gel overnight, rather than at 30 mA for about five hours, as was normally done.

This appears to be a universal property of hsp70, since similar results were obtained by Mitchell et al (1985), in experiments on different larval Drosophila tissues, and mammalian cell lines. They analysed hsp70 on two- and even three-dimensional gels, and found that it was degraded in conditions ranging from those in equilibration buffer, which was neutral and contained 2% SDS, to those in running buffer, which was pH 8.9 and 0.2% SDS.

V.K Expression of hsp70 during unusual heat shock response

Figure V.4 shows the results of an experiment in which the unusual type of heat shock response was obtained after injection of hsp70A DNA (see fig. III.6). One-dimensional gels suggested that hsp70 was synthesized at 20°C (fig. V.4e, track 3) but it was not clear whether it was also synthesized during heat shock (track 6). Two-dimensional gels showed that hsp70, as opposed to proteins 5 and 8, was indeed synthesized at least as efficiently at 35°C as at normal temperatures. However, as the overall rate of protein synthesis was not reduced on heat shock in this experiment, it was not possible to conclude that the mRNA was selectively translated. This confirms that the 70kD proteins are not hsp70, and that they

are not the result of modification of hsp70, because normal, unmodified hsp70 can be synthesized in these oocytes.

F. Discussion

In all the experiments in which synthesis of hsp70 as a result of DNA injection was detected, at least when the heat shock response was 'normal', its RNA was preferentially translated during heat shock. However, there was no evidence that it was masked at normal temperatures. How do these results shed light on the translation of endogenous hsp70 mRNA?

In many species (especially *Drosophila*: Ashburner and Bonner, 1979 and see Chapter I.B.1), hsp70 mRNA is translated much more efficiently than other mRNAs during heat shock. In this respect, the translation of exogenous hsp70 mRNA observed in oocytes reflected this 'classical' behaviour, suggesting that oocytes can translate exogenous RNA appropriately in some circumstances at least, and that the endogenous hsp70 mRNA may be treated in the same way.

The results were not simply a function of the way the RNA was introduced, as when preprochymosin DNA was injected (see Chapter VII for a full discussion), translation of the resulting RNA was always much more efficient at normal temperatures than during a heat shock. This reflected the behaviour of endogenous non-hsp messages.

It has already been demonstrated that RNA transcribed in *Xenopus* oocytes from injected *Drosophila* hsp70 genes during heat shock is only translated after return to normal temperatures (Biens and Pelham, 1982). The results described here support the view that the translation of hsp70 mRNA at

35°C in oocytes is species-specific, since Xenopus hsp70 RNA introduced in the same way was translated during heat shock. It is therefore not surprising that there is no homology between the 5' untranslated region of the Drosophila hsp70 mRNA (Torok and Karch, 1980), which contains translational control signals (McGarry and Lindquist, 1985; Klemenz et al. 1985), and that of Xenopus hsp70 mRNA (Bienz, 1984b).

The question of translational masking is more difficult to resolve. If masking of hsp70 mRNA does not occur in Xenopus oocytes, then it is not surprising that hsp70 was made at 20°C as well as at 35°C: Drosophila hsp70 mRNA is translated efficiently in lysates of non-heat-shocked cells (Storti et al., 1980), so there is no reason to believe that, if the mRNA were actually present in the cells at normal temperatures, it would be translated less efficiently than other mRNA molecules. However, we cannot exclude the possibility that endogenous hsp70 mRNA is masked in oocytes, but the large numbers of RNA molecules transcribed from injected genes within 24 hours could not be handled by the oocytes in the same way as the much smaller numbers of endogenous RNA molecules made over a period of several months.

The preferential translation of hsp70A mRNA transcribed from injected genes described here provided hope that the approach of introducing systematically mutated RNA and following hsp70 synthesis might lead to identification of regions specifying preferential translation, although not of regions specifying masking at normal temperatures.

CHAPTER VI. RESULTS AND DISCUSSION

TRANSLATION OF SYNTHETIC hsp70 RNA IN OOCYTES

VI.A Introduction

The process of identifying translational control regions in hsp70 mRNA would be greatly simplified if RNA itself could be injected directly into oocytes. The amount of RNA introduced could be regulated and would be reproducible, thus eliminating many of the problems encountered with DNA injections, such as variation in transcriptional capacity between batches of oocytes, and low rate of transcription and export to the cytoplasm of hsp70B transcripts.

It is now possible to synthesize RNA in vitro by inserting the desired sequence into vectors containing strong phage promoters, and transcribing with the appropriate enzyme (Melton et al. 1984). A 5' cap structure can be added during transcription, so that the RNA can be translated in vitro and in vivo and is relatively stable in oocytes (Green et al. 1983; Krieg and Melton, 1984; Drummond et al. 1985). These constructs can of course be mutated systematically to produce transcripts with, for example, different 5' untranslated regions.

Because of these advantages, and particularly when it became clear that the exogenous hsp70 RNA which was transcribed in oocytes was regulated appropriately, it seemed worthwhile to investigate whether injected synthetic RNA was subject to the same translational regulation by oocytes. In fact, as discussed in IV.A, it seemed likely that it would not be, since RNA is injected directly into the cytoplasm in large amounts, and not gradually transcribed, processed and exported from the nucleus. Moreover, primary transcripts synthesized

in vitro are never identical to natural mRNA.

In this chapter the construction of a series of plasmids for in vitro transcription is described. These included plasmids encoding a fusion between hsp70 and α -globin (which could be immunoprecipitated) and a truncated hsp70 (which migrated faster than hsp70 in SDS-polyacrylamide gels). Both mutant hsp70s would be detectable without the need for defolliculation and two-dimensional analysis.

Many problems were encountered in detecting translation products after injecting the resulting RNA into oocytes. The basic constructs were, however, gradually refined, to produce ones that gave transcripts as near as possible in structure to the natural hsp70 mRNA, and which were translated in oocytes.

VI.B Subcloning of native and mutant hsp70 sequences into pGEM-2

Initially, the coding sequences for hsp70, the hsp70/globin fusion protein, and a truncated hsp70 protein were transferred to pGEM-2. This vector contains the promoters for the RNA polymerases of the bacteriophages SP6 and T7 at opposite ends of a multiple cloning site. A similar vector, pGEM-1, contains the cloning sites in the opposite orientation. These vectors were based on pSP65 and pSP64 respectively, which contain only the SP6 promoter (Melton et al. 1984).

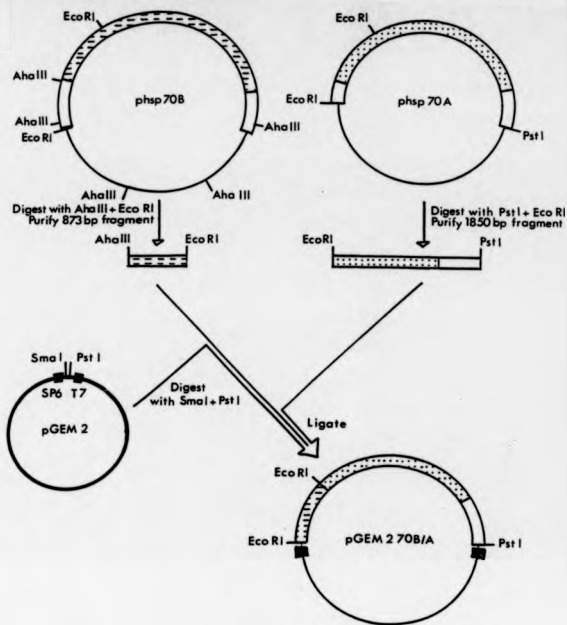


FIG. VI.1a CONSTRUCTION OF pGEM270B/A.

pGEM2 was digested with SmaI and PstI, CIP treated, and ligated with two hsp70 gene fragments: the 873bp AhaIII-EcoRI 5' and fragment of hsp70B, and the approximately 1850bp EcoRI-PstI 3' and fragment of hsp70A (see II.B and II.C for methods).

Stipple, hsp70A; horizontal shading, hsp70B; open boxes, flanking sequences; filled boxes, SP6 and T7 promoters.

VI.B.1 pGEM270B/A

The hsp70 gene inserted into pGEM-2 was actually a chimera, containing the 5' end of the hsp70B mRNA coding sequence fused to the 3' end of hsp70A (fig. VI.1a). This was done because the AhaIII site at position -13 of the hsp70B gene provided a very convenient means of removing upstream sequences so that the RNA transcribed *in vitro* had a 5' untranslated region very similar to the natural message. However, when this work was started, it was not known how similar the coding sequences of the two genes were. The 3' end of hsp70A was therefore used in this construct, so that the construction of genes coding for marked proteins could be planned on the basis of the known sequence and restriction maps of hsp70A (Bienz, 1984b).

The genes were fused at the EcoRI site 860 nucleotides downstream of the cap site, by ligating the AhaIII to EcoRI fragment of hsp70B to the EcoRI to PstI fragment of hsp70A. Since AhaIII leaves a blunt end, the hsp70 gene fragments were ligated with pGEM-2 which had been digested with SmaI (which also leaves a blunt end) and PstI, and treated with CIP. The resulting construct was linearized with PstI and transcribed to give RNA which had 17 nucleotides of vector sequence and the 13 nucleotides of the hsp70B gene immediately upstream of the normal cap site at its 5' end, and approximately 550 nucleotides of genomic sequence downstream of the end of the hsp70A gene (see fig. VI.8).

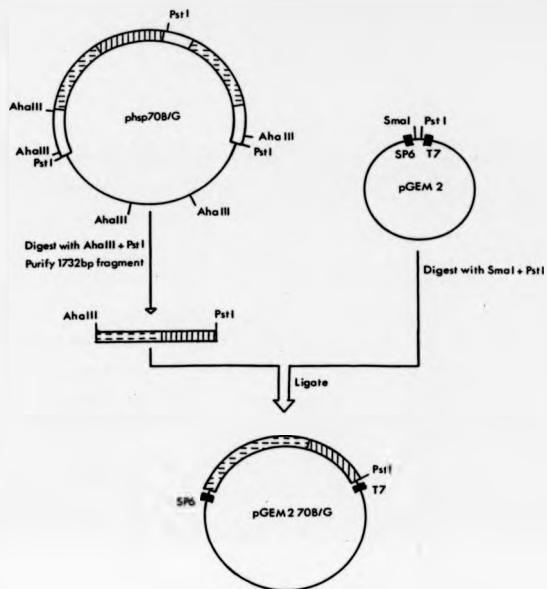


FIG. VI.1b CONSTRUCTION OF pGEM270B/G.

pGEM-2 was digested with *Sma*I and *Pst*I, CIP treated, and ligated with the 1732bp *Aha*III-*Pst*I fragment of phsp70B/G.

Horizontal shading, hsp70B; vertical shading, α -globin; open boxes, ovalbumin or flanking sequences; filled boxes, SP6 and T7 promoters.

VI.B.ii pGEM270B/G

After pGEM270B/A had been constructed I found that, for all enzymes tested the restriction maps of the hsp70A, and hsp70B coding sequences are in fact identical, although the maps of the unsequenced flanking regions (which are also present in the pUC12 plasmids phsp70A and phsp70B) do differ considerably (see fig IV.1b). In the event, therefore, the marked genes were constructed using the hsp70B gene.

The construct pGEM270B/G used for *in vivo* translation studies was the one described in IV.C, designed to check that the fusion of hsp70B to α -globin was in frame: phsp70B/G was digested with AhaIII and PstI and the 1732bp fragment was ligated with pGEM-2 which had been digested with SmaI and PstI and treated with CIP. The RNA transcribed from this construct contained the same 5' end as hsp70B/A, but the 3' end (from the Asp718 site at +1062) was replaced by the α -globin coding sequence (see ^{fig.} VI.8).

VI.B.iii pGEM270B/X

The final construct contained a sequence coding for a truncated form of hsp70B, which, like the fusion gene, allowed injected oocytes to be analysed without defolliculation, since the product migrated faster than native hsp70 in SDS-polyacrylamide gels. This was achieved by inserting a palindromic oligonucleotide containing 'stop' codons in all three reading frames into the XbaI site at +1922. It was thought that this would cause minimal disruption to the RNA structure, a factor which might be important if sequences other than the 5' untranslated region were involved in translational regulation.

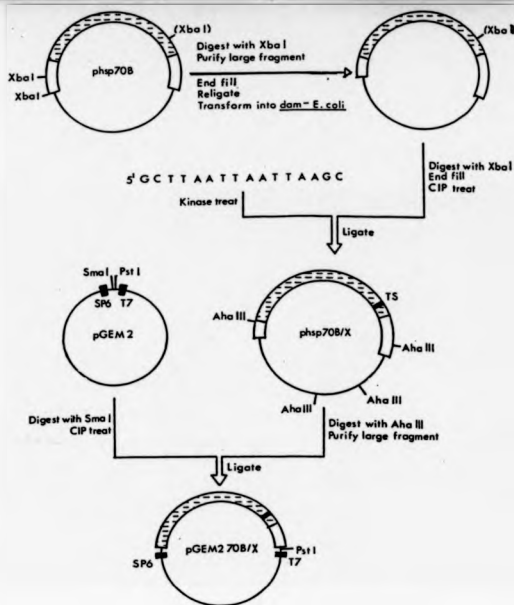


FIG. VI.1c CONSTRUCTION OF pBEM270B/X. After digestion of *pshp70B* with *XbaI*, the large fragment was gel purified, the sticky ends filled in using Klenow enzyme, and the fragment religated. The ligation mix was used to transform *dam⁻ E. coli*. DNA from a transformed colony was linearized with *XbaI*, end filled, CIP treated, and ligated with 'stop' oligonucleotide to generate *pshp70B/X*. This plasmid was digested with *AhaIII*, and the largest fragment, containing the entire coding sequence, was purified. It was then ligated with *pGEM-2*, which had been digested with *SmaI* and *PstI* and CIP treated. Horizontal shading, *hsp70B*; open boxes, flanking sequences; filled box, *SP6/T7* promoters.

This process was not straightforward. Initial restriction digests suggested that the XbaI site was not actually present in the hsp70B gene, so the 'stop' oligo was first inserted into the hsp70A gene, which was then transferred to pGEM-2. However, when RNA transcribed from this construct was translated in vitro, the product was indistinguishable from native hsp70. Further analysis by restriction enzyme digestion revealed that the oligo had in fact been inserted into an XbaI site immediately downstream from the polyadenylation site, in unsequenced DNA (see fig. IV.1b). This suggested that the XbaI site predicted to be present in the gene itself had not been recognized, possibly because it is methylated by E. coli, so cannot be cleaved. Transformation of phsp70A and phsp70B into a dam- strain of E. coli (which lacks the enzymes to methylate XbaI sites) confirmed that this was the case, and that the XbaI site was, after all, present in both genes (data not shown).

A second attempt was made to insert the 'stop' oligo, this time into phsp70B. There are another two XbaI sites in phsp70B, which are unmethylated, and which are both in the upstream flanking region of the gene (see fig. IV.1b), and these were first removed by digestion with XbaI, which produced a large fragment containing the vector and hsp70 coding region, and a smaller fragment containing about 400bp of sequence upstream of the promoter. The large fragment was purified, the sticky ends were filled in using Klenow fragment, and it was self-ligated. The ligation mix was transformed into dam- E. coli. Products of this ligation that were linearized by XbaI had lost the other two sites and were cleaved at the formerly 'cryptic' site. Linearized DNA was end

filled, and ligated with phosphorylated 'stop' oligonucleotide. A transformant (phsp70B/X) which had lost this last XbaI site was selected and digested with AhaIII.

The largest fragment was then purified and ligated with pGEM-2 which had been digested with SmaI and phosphatase treated. However, restriction mapping of the resulting construct showed that, during the insertion of the 'stop' oligonucleotide, about 100 nucleotides of coding sequence around the XbaI site had been lost. Obviously, the loss of sequence would have a greater effect on the structure of RNA transcribed from the construct than insertion of the oligo alone, but the deletion was entirely within the coding region, so the 3' untranslated region was unaffected.

This construct (pGEM270B/X) was linearized for transcription with PstI. The RNA produced had a 5' end identical with the other two RNAs. Apart from the short deletion in the coding sequence, it also contained less 3' flanking sequence than hsp70B/A, as the AhaIII site is about 350 nucleotides downstream of the end of the natural message (see fig. VI.8).

VI.B.iv In vitro and in vivo translation

All three constructs were linearized, transcribed in vitro with SP6 RNA polymerase in the presence of the 'cap' dinucleotide (II.I and II.K), and translated in reticulocyte lysate. All the RNAs were efficiently translated, and gave products readily distinguishable on SDS-polyacrylamide gels (fig. VI.3, tracks 3, 4, and 6). The product of hsp70B/X was about 60kD. As insertion of the 'stop' oligo alone would have produced a polypeptide of about 65kD, it appears that the DNA

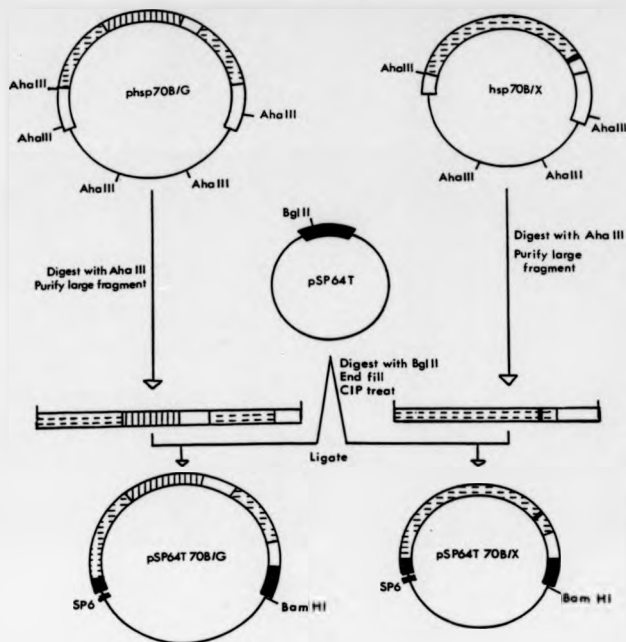


FIG. VI.2 CONSTRUCTION OF pSP64T70B/G AND pSP64T70B/X.

In both cases, this was done by digesting the corresponding pUC12 construct (pSP64T70B/G or pSP64T70B/X) with Aha III, and purifying the largest fragment. This was then ligated with pSP64T, which had been digested with Bgl II, and filled and CIP treated. Horizontal shading, hsp70B; vertical hatching, α -globin sequences; filled boxes, β -globin sequences and SP6/77 promoters.

deleted was immediately 5' to the insertion site.

These RNAs were injected into oocytes on several occasions, but no in vivo translation products were ever detected. This was particularly surprising as one of these occasions was the only experiment in which the hsp70B/globin fusion was detected after injection of phsp70B/G DNA (see fig. V.1).

VI.C Subcloning of mutant hsp70 sequences into pSP84T

There were many possible explanations for the failure to detect in vivo translation products of the SP6 RNAs described above. One of the most plausible, and the simplest to rectify, was that it was due to their lack of poly(A) tails: work in this laboratory indicated that translation of poly(A)- SP6 RNAs in oocytes is less efficient than translation of poly(A)+ RNAs and that the addition of a poly(A) tail to synthetic RNA increases both its stability and its translatability (Drummond et al. 1985). Obviously, both these factors have implications for the amount of protein produced by injected RNAs.

SP6 RNAs can be provided with functional 5' and 3' untranslated regions including a poly(A) tail by using the vector pSP84T (Krieg and Melton, 1984). In addition to the SP6 promoter, this contains the 5' and 3' untranslated regions of a *Xenopus* β -globin cDNA clone, separated by a *Egl*II site into which the appropriate sequences are inserted (fig. VI.2). At the 3' end of the globin sequence is a stretch of 23 dA-dT residues, followed by about 30 dC-dG residues resulting from the GC tailing used to clone the cDNA (Williams et al. 1980).



FIG. VI.3 IN VITRO TRANSLATION OF SYNTHETIC RNAs.

Reticulocyte lysate translations (II.K.1) were carried out with the following additions: track 1, no RNA; 2, oviduct poly(A)+; 3, pBEM270B/A RNA; 4, pBEM270B/X RNA; 5, pSP64T70B/X; 6, pBEM270B/G; 7, pSP64T70B/G. All synthetic RNAs were transcribed using SP6 RNA polymerase, and in each case 100ng RNA was added to a 10 μ l translation mix.

Both the mutant hsp70B genes described above were transferred to this vector (fig. VI.2). In both cases, this was done by excising the entire coding sequences plus about 350bp of downstream flanking sequence with AhaIII, and ligating it to pSP64T which had been digested with BglII, end filled, and phosphatase treated.

After linearization with PstI, the constructs were transcribed to give capped RNAs containing the coding sequences of the mutant genes flanked by the β -globin 5' and 3' untranslated regions (see fig. VI.8). An important difference between the transcripts of pSP64T70B/G and pGEM270B/G was that the former contained all of the hsp70 coding sequence, not just the sequence 5' to the fusion point. Its structure was therefore nearer to that of the RNA transcribed when phsp70B/globin DNA was injected into oocyte nuclei.

When these RNAs were translated in reticulocyte lysate, the products were indistinguishable from the products of the original pGEM-2 constructs, as expected (fig. VI.3, compare tracks 4 and 5, and 6 and 7). However, when they were injected into oocytes, no translation products were detected, despite the presence of a poly(A) tail.

VI. D Site-directed mutagenesis

The AhaIII site in the hsp70B gene was used for insertion into SP6 vectors because it is only 13 nucleotides upstream of the cap site in the natural mRNA. However, only seven nucleotides upstream of the cap site is an ATG triplet, followed three later by a TAG 'atop' codon (see fig. IV.1a).

These were of course present in the SP6 RNAs transcribed from all the constructs described above. The rules governing usage of initiation codons by eukaryotic ribosomes are complex, but they suggested that the presence of this short open reading frame upstream of the correct initiator would not prevent translation of hsp70 from the SP6 RNAs, although it might reduce its efficiency.

In general, initiation of translation occurs at the AUG nearest the 5' end of the RNA, unless it is in a 'poor context', in which case initiation at a downstream AUG is favoured. Analysis of known mRNA sequences, confirmed by mutational studies, has identified the optimal sequence context for initiation as (GCC)GCCACCAUGG (Kozak, 1984a, 1986, and 1987). The presence of a purine at position -3 is dominant, so that point mutations at the other positions, particularly -1 and -2, only have a significant effect if there is a pyrimidine at -3. There are a few examples of mRNAs in which initiation occurs at an internal AUG, even when the upstream AUG is in an excellent context, but this depends on the presence of a 'stop' codon in frame with the first AUG and upstream of the second (Kozak, 1984b). However, the presence of an upstream open reading frame does reduce the efficiency of initiation at the correct position (Kaufman *et al.* 1987).

The upstream AUG in the hsp70 SP6 constructs is in the sequence CCCAAAGCGAUGU. and the natural hsp70B RNA initiator AUG is in AGCGCAAUAUGG. Neither of these matches the consensus sequence perfectly. The correct AUG, however, coincides at the two most important positions (-3 and +4). It therefore appeared that initiation at the correct triplet would probably be favoured in this case. Moreover, the

presence of an in-frame 'stop' codon upstream of the correct AUG suggested that hsp70 would be translated from the SP6 RNAs even if the upstream AUG was used. The fact that they were translated apparently normally *in vitro* encouraged the belief that they would be translated in oocytes.

It was therefore surprising that translation products were not detectable in oocytes, even when the RNAs were provided with a poly(A) tail. However, the difficulties encountered in detecting expression of the hsp70B/globin from RNA transcribed from the correct cap site after DNA injection into oocytes (section V.2) suggested that additional factors might be acting to bias against the detection of any mutant hsp70 peptides; they might be unstable, for example. An alternative explanation might be that the problems were related to the hsp70B 5' untranslated region, since this is slightly different from that of hsp70A. In either case any reduction in translational efficiency due to the presence of the upstream AUG might, in combination with these other factors, mean that amounts of mutant hsp70 remained below the limit of detection.

It was therefore decided to remove the upstream ATG from the SP6 constructs by site-directed mutagenesis. In fact, this was done by introducing a Sali site (and therefore HincII and AccI sites) directly over the natural hsp70 cap site, and using this new site to subclone into SP6 vectors (fig. VI.4). This approach had two advantages. Firstly, the same procedure could be used to mutate the hsp70A and hsp70B genes, allowing an investigation of possible differences in translational behaviour of the two RNAs. Secondly, the extraneous sequences at the 5' end of the SP6 RNAs would be further reduced.

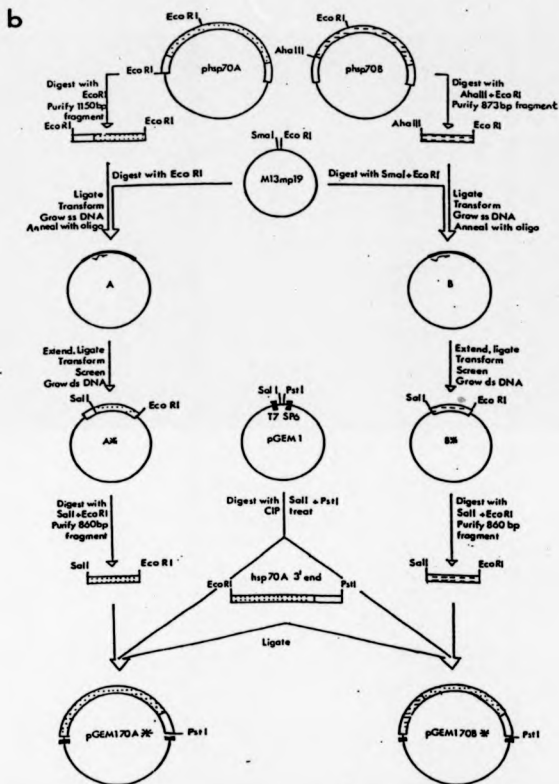
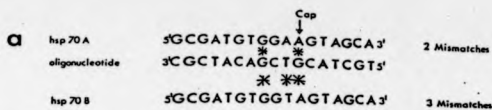


FIG. VI.4 IN VITRO MUTAGENESIS.

(a) Sequence of the oligonucleotide and the complementary region of the hsp70A and hsp70B genes. Asterisks denote mismatches between each gene and the oligonucleotide; the position of the natural cap site is indicated.

(b) The 5' end of the hsp70A gene was subcloned into M13p19 by digesting phsp70A with EcoRI, purifying the 1150bp fragment, and ligating it with M13p19 which had also been digested with EcoRI. The 5' end of the hsp70B gene was subcloned by digesting phsp70B with AhaIII and EcoRI, purifying the 873bp fragment, and ligating it with M13p19 which had been digested with SmaI and EcoRI. The two constructs were subsequently treated identically: the ligation mixes were used to transform E. coli TB2 cells. Single stranded phage DNA was purified from recombinant plaques, and this was sequenced by the dideoxynucleotide chain termination method using the autagenic oligonucleotide as a primer, to confirm correct orientation of the inserts. Aliquots of correct single stranded DNA preparations were then annealed with the oligonucleotide, and the second strand was synthesized by incubating the annealed DNA in the presence of the four dNTPs, Klenow enzyme and T4 DNA ligase. The reaction mixes were used to transform E. coli BMH71:18 mutL cells (which are deficient in mismatch repair) grown on a lawn of BMH71:18 cells.

Transformants were screened by hybridization to the radioactively labelled oligonucleotide. Double stranded DNA was prepared from positive plaques and digested with SmaI and EcoRI. The resulting 860bp 5' and fragments were ligated with the EcoRI-PstI 3' end fragment of hsp70A, and pGEN-1 which had been digested with SmaI and PstI and CIP treated. This generated the plasmids pGEN170A+ and pGEN170B+. Full details of the methods used are given in II.8.

Stippled boxes, hsp70A transcribed sequences; horizontal shading, hsp70B sequences; open boxes, flanking sequences; filled boxes, SP6/T7 promoters.

The mutagenesis procedure is discussed in detail in II.G, and an outline of the principle is given in figure VI.4. In brief, an oligonucleotide was designed to act as a primer for mutagenesis. It was a 16-mer, which annealed to hsp70A with two mismatches, and to hsp70B with three mismatches. Sequences spanning the cap site were excised from the hsp70 genes and transferred into the M13 plasmid mp19. For hsp70A, the smaller EcoRI fragment was used, and for hsp70B an AhaIII-EcoRI fragment was used. Single stranded DNA was grown up and sequenced using the mutagenesis primer. This served two purposes: it confirmed the inserts were in the correct orientation and also showed that the primer annealed only at the intended point, since the resulting sequence was unambiguous (data not shown).

The DNAs were then annealed to the primer, and the second strand was synthesized by extension with Klenow fragment and ligated. The ligation mixture was transformed into BMH 71:18 mut.L cells, which are deficient in mismatch repair. 84 plaques from each of the two reactions (hsp70A and B) were screened by growth as colonies on a grid. These were lifted onto nitrocellulose and hybridized with radioactive mutagenesis primer. Initially this hybridized to all the colonies, but as the temperature of the 6XSSC wash solution was increased to the T_m for the oligo, the radioactive probe dissociated from the wild type DNA, but remained annealed to the mutant. The reaction with hsp70A yielded 41 positive colonies, and that with hsp70B yielded 24, presumably reflecting the greater number of mismatches. Confirmation that mutagenesis had occurred was obtained by subsequent digestion of the DNA at the new Sali site, and double-stranded

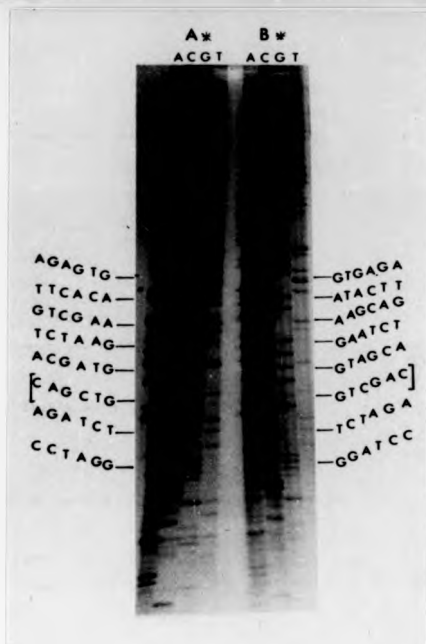


FIG. VI.5 SEQUENCE OF pBEM170A* AND pBEM170B*.

The plasmids were sequenced by the method described in II.E.ii, using 2µg of DNA and the primer that binds to the T7 promoter. Reactions were analysed on a 6% acrylamide denaturing gel. Fragments representing the SalI site are bracketed; fragments shorter than these represent nucleotides in the polylinker of pBEM1, larger fragments represent hsp70 sequences.

sequencing of the construct used for in vitro transcription (see below).

VI.E Subcloning of hsp70* sequences into pGEM-1

After plaque purification, double stranded DNA was grown up from positive colonies selected at random from the mutagenesis procedure. Using the new Sall site and the original EcoRI site, DNA corresponding to the 5' end of the hsp70A and B RNAs was excised (see fig. VI.4). These fragments were ligated to the EcoRI-PstI fragment of hsp70A (which encodes the carboxyl end of the protein), and to pGEM-1 which had been digested with Sall and PstI and phosphatase treated. The constructs formed were pGEM170A* and pGEM170B*. The very presence of the Sall site indicated that mutagenesis had been successful in the plaques selected. However, further unequivocal evidence was given by sequencing of the join between the Sall sites of pGEM-1 and the hsp70 genes using a primer that annealed to the T7 promoter (fig VI.5).

In this case sequences coding for native hsp70 were used for in vitro RNA synthesis, because of the difficulties encountered in detecting expression of the truncated and fusion proteins in oocytes. After linearization with PstI, the constructs were transcribed with T7 polymerase, since they were in pGEM-1, in the presence of the alternative cap analogue (GpppG). This dinucleotide does not by itself inhibit translation, so the transcription mix could be translated in vitro and in vivo without purification of the RNA by repeated ethanol precipitation (II.I.1). The structures of the RNAs are shown schematically in figure VI.8.

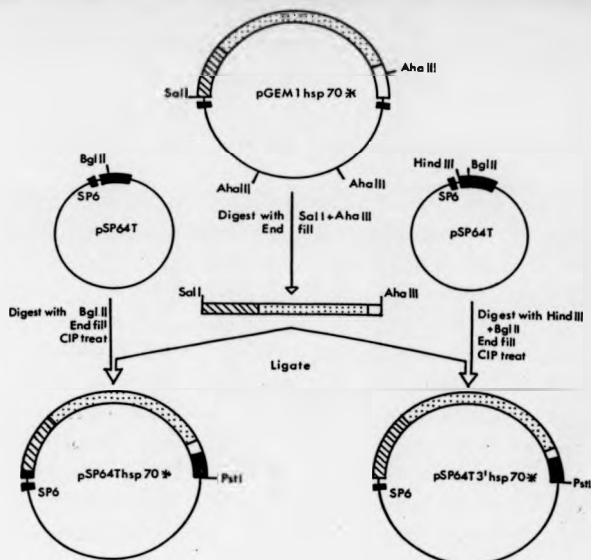


FIG. VI.4 TRANSFER OF hsp70A* AND hsp70B* INTO pSP64T.

The pSP64T vector was prepared in two different ways: by linearizing with BglII, and filling with Klenow, and CIP treating; or by digesting with BglII and HindIII (which removes the β -globin 5' untranslated region) then treating with Klenow and CIP as before. The hsp70 coding sequences were excised from pGEN170A* and pGEN170B* using SalI and AhaIII. Each was then ligated with each of the vector preparations to produce the constructs pSP64T70A*, pSP64T70B*, pSP64T3'70A* and pSP64T3'70B*.

Hatched boxes, hsp70A* and hsp70B* DNA; stippled box, hsp70A DNA (3' end of gene); filled boxes, T7 and SP6 promoters, β -globin 5' and 3' untranslated regions; open boxes, flanking sequences.

However, despite the removal of the upstream AUG, and the fact that they were translated in vitro (data not shown), translation products of these RNAs were yet again undetectable after injection of the RNA into oocytes. This was perhaps not surprising given that no attempt had been made to provide these RNAs with poly(A) tails (see VI.C).

VI.F Subcloning into pSP64T and expression in oocytes of hsp70B/A, hsp70A* and hsp70B*

VII.F.i Subcloning

To provide the synthetic RNAs with poly(A) tails, the coding sequences were transferred into pSP64T, as before (see VI.C). The large Sall-AhaIII fragment was removed from pGEM170A* and from pGEM170B* (fig. VI.6) and ligated with BglII-out, phosphatase-treated pSP64T. This generated the constructs pSP64T70A* and pSP64T70B*. In addition, to assess the effect of removing the upstream AUG, the AhaIII-EcoRI fragment at the 5' end of hsp70B and the EcoRI-AhaIII fragment at the 3' end of hsp70A were also ligated into pSP64T, to generate pSP64T70B/A (construction not shown).

Since the hsp70 RNAs do contain their own functional 5' untranslated regions, and addition of extraneous sequences to this region of Drosophila hsp70 mRNA caused preferential translation to be lost (McGarry and Lindquist, 1985), a further two constructs were created which lacked the 5' untranslated sequences of the globin message. This was done simply by digesting pSP64T with HindIII and BglII, then end filling, phosphatase treating and ligating to the fragments containing hsp70A* and hsp70B* (fig. VI.6). The resulting plasmids were pSP64T3'70A* and pSP64T3'70B*.

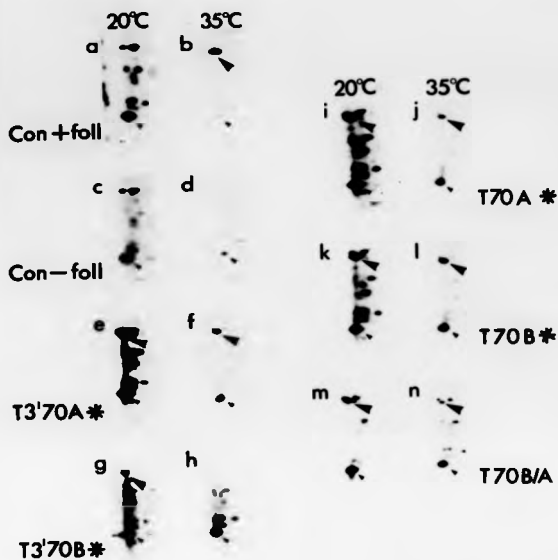


FIG. VI.7 INJECTION OF SP64T-DERIVED hsp70 RNA INTO OOCYTES.

SP6 RNA was injected into oocytes (about 4ng/oocyte), which were then incubated at 20°C overnight before being labelled for 2h in MBS containing 1mCi/ml [³⁵S]methionine at either 20°C or 35°C. Surviving oocytes were defolliculated, then analysed by two-dimensional SDS-PAGE. Half an oocyte-equivalent was loaded per gel. Controls were uninjected oocytes treated in the same way: panels a and b, controls with follicle cells incubated at 20°C and 35°C respectively; c and d, controls defolliculated before analysis; e and f, injected oocytes, RNA derived from pSP64T3'70A; g and h, RNA derived from pSP64T3'70B; i and j, pSP64T70A; k and l, pSP64T70B; m and n, pSP64T70B/A. Large arrow, hsp70; small arrow, actin.

VI.F.ii Expression

These constructs were linearized with either *Pst*I or *Bam*HI, and capped transcripts synthesized *in vitro* using GpppG (see fig. VI.8). When the transcription mixes were injected into oocytes, translation products were detected in all five cases, in oocytes incubated both at 20°C and during a heat shock (fig VI.7). Translation was, however, reduced by a heat shock in all cases. Other proteins migrating in the same region of the gels as hsp70 make it difficult to assess whether there are slight differences in the expression of the five RNAs injected. It seems, though, that none is preferentially translated.

Because of the earlier failure to detect synthesis of mutant hsp70s from constructs containing the upstream AUG, it was surprising that all these RNAs were translated well at 20°C, including the one that contained the AUG upstream of the hsp70 initiator codon (panels m and n). It seems that the failure to detect translation of the earlier SP8 RNAs was due more to the use of mutant coding sequences than to the presence of the upstream AUG.

It was not altogether unexpected that the RNAs with the globin 5' untranslated leader were translated poorly during a heat shock, despite being translated efficiently at 20°C (panels i-n): this would be predicted by the results of McGarry and Lindquist (1985), discussed above (I.B.ii). It was disappointing, however, that even when the globin leader was removed, preferential translation was not observed (e-h).

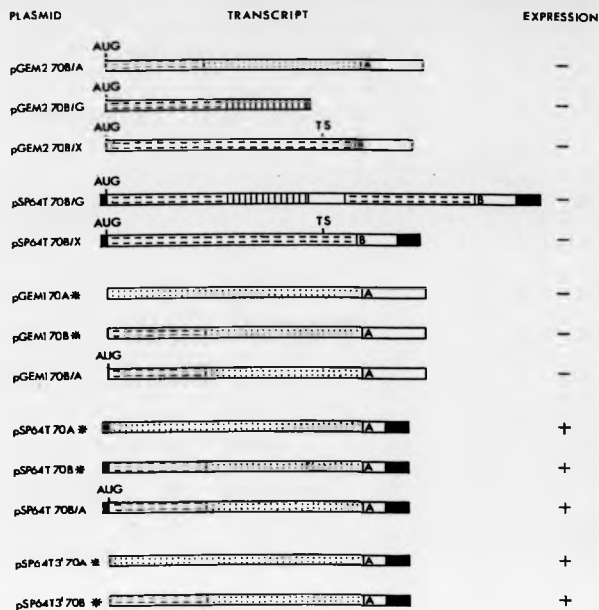


FIG. VI.8 STRUCTURES OF SYNTHETIC hsp70 RNAs.

Stipple, hsp70A RNA; horizontal shading, hsp70B; vertical shading, α -globin; open boxes, hsp70 3' flanking regions or ovalbumin DNA (pSP64T70B/B only); filled boxes, β -globin 5' and 3' untranslated regions; TS, translational stop oligonucleotide; AUG, translation initiator codon upstream of natural initiator; +/- indicates whether translation of each RNA was detected in injected oocytes.

VI.G Discussion

The results described illustrate the difficulties encountered in using SP6 RNAs to study translational control in vivo. Firstly, it is impossible to synthesize RNA in vitro which is identical to endogenous message. Transcription of the constructs pSP64T3'70A* and pSP64T3'70B* yielded RNAs nearest in structure to the natural message (see fig. VII.8), but even these transcripts were not translated appropriately by the oocytes. Results presented in the following chapter suggest that this may be a general problem when RNA is injected. Secondly, detection of translation products from transcripts coding for mutant hsp70 proved to be impossible. The fusion protein was immunoprecipitated very inefficiently, but both it and the truncated hsp70 should have been visible in the total homogenates, if the RNAs were translated as well as those coding for native hsp70. (The constructs encoding these proteins contained an upstream ATG, but native hsp70 was synthesized from similar transcripts.) This suggests that the products were unstable in oocytes.

The potential advantages of the use of RNA, rather than DNA, to study translational control of hsp70 mRNA in oocytes have not therefore been realised, because of these practical problems. It appears that injection of hsp70 DNA will be the only viable approach despite the difficulties of controlling the amount of RNA transcribed and exported to the cytoplasm. However, it will still be necessary to construct a gene for a mutant hsp70 which can be expressed, since analysis of large numbers of samples by two-dimensional SDS-PAGE is both difficult and laborious.

CHAPTER VII: RESULTS AND DISCUSSION

EXPRESSION OF PFKFROCHYMOBIN AND FLU NUCLEOPROTEIN DURING HEAT SHOCK

VII.A Introduction

In the early experiments investigating expression by oocytes of injected hsp70 genes, a control DNA known to be efficiently expressed was also injected. This was done because the capacity of oocytes to transcribe injected DNA and to translate the resulting RNA varies considerably between batches. Failure to detect hsp70 protein after injection of hsp70 genes could therefore obviously be due to a generalized inability to transcribe or translate any exogenous DNA, rather than to a specific inability to translate hsp70 mRNA. Moreover it was possible that any apparent translational control could be due to the method of introduction of the genes, rather than to specific control sequences in the mRNA.

The construct pTKNP (Davey et al., 1985a) contains the influenza virus NP gene inserted into pTK-. In this vector, the gene of interest is inserted between sequences containing the promoter and 5' untranslated region of the herpes simplex virus thymidine kinase (TK) gene, and the termination and polyadenylation signals of the SV40 early transcripts (Kreig et al., 1984). This construct seemed an ideal choice as a control DNA in experiments designed to study expression of hsp70. Firstly, it is very efficiently transcribed in oocytes, yielding RNA with the 5' leader of the TK gene followed by the NP leader and coding sequence. Its translation product may represent up to 7% of the total protein synthesized in injected oocytes at normal temperatures (Davey et al., 1985a and b). Secondly, Pelham and Bienz (1982) had demonstrated

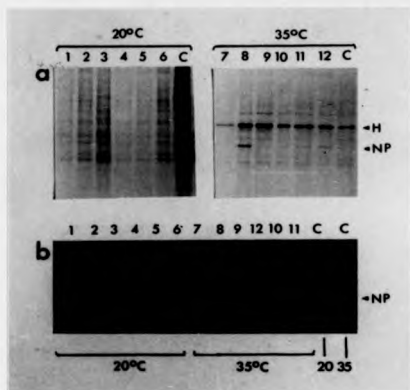


FIG. VII.1 EXPRESSION OF pTKNP DURING HEAT SHOCK IN OOCYTES.

After injection of 2ng pTKNP DNA per oocyte, followed by overnight incubation at 20°C, oocytes were incubated in MBS containing 1aCi/ml [³⁵S]methionine for 2h at either 20°C or 35°C. Surviving oocytes were analysed individually on 12.5% SDS-acrylamide gels as total homogenates (panel a) or after immunoprecipitation using the monoclonal anti-NP 5/1 (panel b). Tracks 1-6, injected oocytes labelled at 20°C; 7-12, injected oocytes labelled at 35°C; C, uninjected controls. Panel a, one eighth of an oocyte per track; panel b, one quarter of an oocyte per track. NP is indicated with an arrow; hsp70, H.

that transcription from the TK promoter is inhibited about fivefold in oocytes during heat shock, so that the levels of RNA present before and during heat shock would be expected to be very similar.

It was anticipated that the protein would be produced only at normal temperatures. Surprisingly, in the first experiment, NP was expressed more efficiently during heat shock than at normal temperatures. This chapter describes a series of experiments to investigate this finding further. It was hoped they would provide a short cut to identifying the sequence allowing selective translation of specific mRNAs during heat shock.

VII.B Expression of NP during heat shock

VII.B.1 Patterns of NP expression

The results of the first experiment were completely unexpected. Figure VII.1a shows SDS-PAGE analysis of individual oocytes injected with pTKNP DNA, incubated at 20°C overnight, then radioactively labelled for two hours at either 20°C (tracks 1-6) or 35°C (tracks 7-12). NP is clearly visible in several tracks even without immunoprecipitation, and is more prominent in heat shocked than non-heat-shocked oocytes - in track 8, for example. Immunoprecipitation of these samples confirmed this result (fig. VII.1b).

As the follicle cells were not removed in this experiment, the level of protein synthesis in the oocytes themselves could not be determined. However, the overall level of protein synthesis fell by at least 30% in these oocytes, whereas the level of NP synthesis was slightly increased by heat shock. Since the follicle cells always contribute a

larger proportion of the overall protein synthesis during heat shock than at normal temperatures (see III.D), then this must under-estimate the preferential translation of NP during heat shock.

The analysis of individual oocytes illustrated here also dramatically demonstrates the variability, both in overall level of protein synthesis and particularly in expression of the exogenous DNA, between oocytes which had been treated identically. This phenomenon posed a significant problem, since it was not practical to analyse large numbers of oocytes individually. In any case, it was not known if the failure of particular oocytes to produce NP was due to a failure of transcription or of translation, or whether the DNA injected had simply missed the germinal vesicle. It would therefore have been difficult to interpret the results even of individual analyses. In most subsequent experiments, therefore, the samples were made as large as possible, and all oocytes in each sample were analysed together.

Although results similar to those described above were found on several occasions, several other patterns of expression of NP were also observed, and these are illustrated in figure VII.2. In all these experiments, oocytes were radioactively labelled at normal temperatures, during a single heat shock, or during a second heat shock administered after a two hour recovery period. In some experiments, oocytes were also labelled at 20°C for two hours, after having been allowed to recover from heat shock for two hours.

In one case, in which oocytes were analysed individually, NP was barely detectable in oocytes labelled at 20°C (fig. VII.2a, tracks 1-3). A small amount was found in

oocytes given a single heat shock (tracks 4-8), but significant amounts of NP were only made during the second heat shock (tracks 7-8). In fact, two bands corresponding to proteins of very similar molecular weight were visible in the immunoprecipitated samples. NP was the lower band. The upper band was only seen when, as in this experiment, the oocytes mounted the more unusual type of response (see III.F). As it was much more prominent in heat shocked oocytes, it was probably due to some or all of the induced 80kD proteins binding non-specifically to the rabbit antibody. When the monoclonal antibody was used (see, for example fig. VII.2b) the band was not seen, but it was always seen when rabbit antibody was used (see, for example, fig. VII.4a).

In another experiment, when batches of oocytes were analysed, a similar pattern was obtained: NP was produced during both the first and second heat shocks but not either before heat shock or during recovery (fig. VII.2b). In a third (fig. VII.2c), the exact reverse was true: NP was produced only at 20°C and during recovery.

In other words, NP RNA transcribed in oocytes behaved in different batches of oocytes in one of three ways: firstly, in the same way as hsp70 mRNA transcribed from injected genes (fig. VII.1), in that it was translated at 20°C but was preferentially translated during heat shock; secondly, in a manner apparently analogous to the behaviour of 'masked' messages, in that it was translated much more efficiently during heat shock than at normal temperatures (fig. VII.2a and b); and finally as a normal 20°C message (fig. VII.2c). There was no obvious reason for these observed differences, although the behaviour of the NP RNA appeared to be related to the type

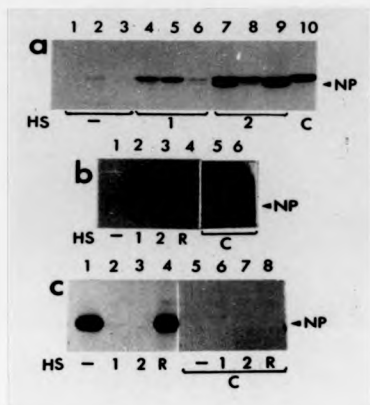


FIG. VII.2 DIFFERENT PATTERNS OF pTKNP EXPRESSION.

In each experiment, 2ng pTKNP DNA was injected per oocyte. After overnight incubation at 20°C, oocytes were labelled in MBS containing 1mCi/ml [³²S]methionine for 2h in four different conditions: at 20°C, 35°C, during a second heat shock after a 2h period at 20°C, or during the second 2h of recovery at 20°C after a heat shock. Oocytes were then analysed by immunoprecipitation followed by electrophoresis on 12.5% SDS-polyacrylamide gels.

Panel a: individual oocytes labelled at 20°C (tracks 1-3), during the first heat shock (4-6), and during the second heat shock (7-9); the uninjected control (10) was labelled during the second heat shock. Samples were immunoprecipitated using the rabbit anti-influenza antibody; one quarter of an oocyte loaded per track.

Panel b: oocytes labelled at 20°C (track 1), during the first heat shock (2), during the second heat shock (3), and during recovery (4). Uninjected controls were incubated at 20°C (5) and during the first heat shock (6). Five oocytes pooled per sample; immunoprecipitated using the monoclonal antibody 5/1; 0.75 oocyte equivalents per track.

Panel c: injected (tracks 1-4) and control (5-8) oocytes were labelled at 20°C (1 and 5), during the first heat shock (2 and 6), during the second heat shock (3 and 7), and during recovery (4 and 8). 14-20 oocytes pooled per sample; immunoprecipitated using the monoclonal antibody 5/1; 1.25 oocytes per track.

of heat shock response elicited in the oocytes, which in turn is probably determined by some environmental factor (see III.F).

In general, in experiments in which NP mRNA was apparently partially masked at 20°C (fig. VII.2a and b), the heat shock response was of the more unusual type - the synthesis of specific proteins was induced in the oocytes and the overall level of protein synthesis did not fall greatly (see III.F). Moreover, during the second heat shock, when expression of NP was usually greatest, the reduction in the level of protein synthesis was less than during the first heat shock. However, in all the cases illustrated here, the overall level of protein synthesis was somewhat reduced during both heat shocks, compared with the level at 20°C. The NP RNA was therefore translated preferentially during the heat shocks. In these cases the behaviour of NP RNA resembled that of the RNA encoding those endogenous oocyte proteins whose synthesis was dramatically increased on heat shock (when the unusual response occurred).

In contrast, when the NP RNA behaved in the same way as RNA transcribed from injected hsp70 genes (i.e. it was not masked at normal temperatures, and its translation was maintained during heat shock; fig. VII.1), the oocytes generally mounted the 'normal' type of heat shock response. The occasions when NP RNA behaved like a typical endogenous 20°C RNA were not particularly associated with either type of heat shock response. In these cases, the reduction of translation of NP RNA during heat shock was not due to degradation of the RNA, as it was efficiently translated on return to normal temperatures (fig.VII.2c, track 4).

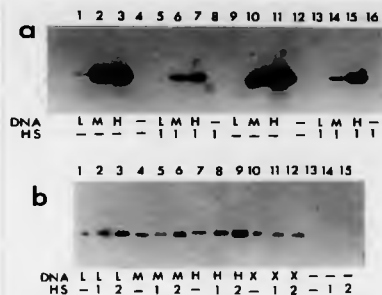


FIG. VII.3 EFFECT ON EXPRESSION OF pTKNP OF AMOUNT OF DNA INJECTED.

Panel a: 0.1ng (L), 1ng (M), or 2ng (H) DNA was injected per oocyte into oocytes from two frogs (frog 1, tracks 1-8; frog 2, 9-16). After overnight incubation at 20°C, the oocytes were labelled as normal either at 20°C (HS-) or during the second heat shock (HS1); control uninjected oocytes, tracks 4, 8, 12, and 16. Each sample contained 9-24 oocytes pooled; the gel shows immunoprecipitations (using the monoclonal antibody 5/1); 1.25 oocytes loaded per track.

Panel b: 0.5ng (L), 1ng (M), 2ng (H), or 0.75ng (X) DNA was injected per oocyte. After overnight incubation oocytes were labelled as normal at 20°C (HS-), or during the first (HS1) or second (HS2) heat shocks; control uninjected oocytes, tracks 13-15. Samples each contained 8-13 oocytes; the gel shows immunoprecipitations (using the monoclonal antibody 5/1); 0.25 oocytes per track.

VII.B.ii Effect of DNA concentration

A possible explanation for these great differences in the behaviour of NP RNA was that oocytes contained a factor involved in the preferential translation of NP RNA, which was present at widely differing concentrations in the oocytes of different frogs. If so, we predicted that it should be possible to mimic the whole range of effects by varying the amount of RNA present in oocytes from the same frog. This can be achieved by injecting DNA at a range of concentrations, although the amount of RNA transcribed does not increase proportionally with the concentration of DNA injected (Davey, 1984). In different batches, the concentration of DNA producing enough RNA to saturate the factor would vary.

However, when this experiment was carried out on oocytes from three different frogs, the concentration of pTKNP DNA did not appear to affect the way resulting NP RNA was translated. Oocytes from frogs 1 and 2 (fig. 3a) were labelled at 20°C or during the second two-hour heat shock. They always treated NP RNA like a normal RNA: much less NP was synthesized during heat shock for all concentrations of DNA tested. The amount of NP made did increase with DNA concentration, indicating that the amount of RNA transcribed was indeed related to the concentration of DNA injected. In contrast, oocytes from a third frog translated the NP RNA with roughly the same efficiency at either temperature and at all concentrations of DNA (fig. VII.3b). In this case, however, the amount of protein produced did not increase greatly with DNA concentration, so it is possible that the range of RNA concentrations was not so wide as in oocytes from frogs 1 and

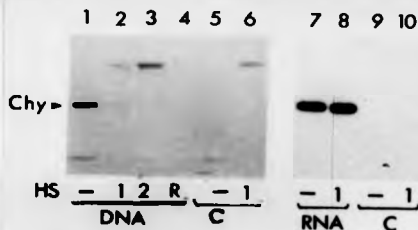


FIG. VII.4 EXPRESSION OF pTKB2 AND STABILITY OF PREPROCHYMOSIN IN OOCYTES.

After injection of 4ng pTKB2 per oocyte, oocytes were incubated at 20°C overnight, then labelled for 2h as usual. Samples (each containing 5 oocytes) were analysed by immunoprecipitation using the rabbit anti-prochymosin antibody, followed by electrophoresis on a 12.5% SDS-polyacrylamide gel; 0.75 oocyte was loaded per track. Injected oocytes were labelled at 20°C (track 1), during the first (2) or second (3) heat shock, or during recovery (4). Control uninjected oocytes were labelled at 20°C (5), or during the first heat shock (6).

To test the stability of preprochymosin, after injection of 2ng preprochymosin mRNA per oocyte, oocytes were incubated at 20°C overnight, then incubated for 4h in MBS containing 1mCi/ml [³⁵S]methionine. They were transferred to unlabelled MBS, and incubated for a further 2h at 20°C (track 7) or 35°C (track 8). Homogenized, pooled oocytes were then analysed by immunoprecipitation and SDS-PAGE as before. Control oocytes were labelled, then incubated at 20°C (track 9) or 35°C (track 10) and analysed in the same way.

2.

Despite this, the failure to alter the pattern of NP expression, even when a wide range of RNA concentrations was inferred, indicates that the differences in the treatment of NP RNA by different batches of oocytes are not due simply to the presence of different levels of a factor involved in preferential translation during heat shock.

VII.C Expression of chymosin during heat shock

Two explanations that do not invoke specific translational control are possible for the efficient expression of NP during heat shock in some oocytes. Firstly, transcription from the TK promoter might actually be induced by heat shock. Secondly, it may be that, for some unknown reason, all RNA transcribed from genes injected into oocytes can be preferentially translated. These possibilities were tested by injecting groups of oocytes from the same frog with either pTKNP or pTK82 (a construct containing cDNA encoding calf preprochymosin in the pTK vector). The oocytes were incubated overnight at 20°C then labelled at 20° or 35°C as normal. Preprochymosin was invariably synthesized much more efficiently at 20°C than during heat shock, even when NP was synthesized more efficiently during heat shock in oocytes from the same batch (fig. VII.4 tracks 1-6, which show the same experiment as fig. VII.2.b). Note that the faint band corresponding to protein of about 80kD was again precipitated in this experiment, when the rabbit preprochymosin antibody was used.

To check that the reduced production of chymosin during heat shock was not simply a result of degradation of the protein during heat shock, natural preprochymosin mRNA was injected into oocytes. These were labelled at 20°C for four hours, then incubated without label for two hours at either 20° or 35°C (fig. VII.4, tracks 7 and 8). Clearly, a heat shock did not reduce the amount of radioactive preprochymosin in the oocytes (compare tracks 7 and 8). The polypeptide must therefore be stable during heat shock.

Since the promoters in pTK82 and pTKNP are identical, their patterns of transcription at normal temperatures and during heat shock would be expected to be similar. If so, the different patterns of expression of the two proteins must be due to differences in either the translatability or the stability of the two RNAs. Since RNA which is translationally repressed during heat shock is not normally degraded (see e.g. DiDomenico *et al.* 1982b), the latter possibility seems unlikely. However, it cannot be excluded because of the unusual way in which the RNA was introduced into the oocytes. Moreover, preprochymosin was not efficiently expressed during recovery (fig. VII.4a, track 4), suggesting that, in this experiment at least, the repression of translation may not have been reversible.



FIG. VII.5 COINJECTION OF pTK82 AND pTKNP.

A mixture containing 100ng/ μ l of each DNA was injected, so that each oocyte received 2ng of each DNA. After overnight incubation at 20°C, they were labelled for 2h at 20°C (HS-), during the first (HS1) or second (HS2) heat shock, or during recovery (HSR). Surviving oocytes (7-10 oocytes per sample) were homogenized and immunoprecipitated with the monoclonal anti-NP 5/1 (tracks 1-4) and rabbit anti-prochymosin (tracks 5-8), separately. Control, uninjected oocytes (tracks 9-12) were treated in the same way, except that both antibodies were added to the same immunoprecipitation reactions.

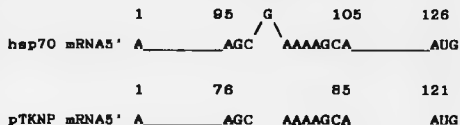
VII.D Coinjection of pTKNP and pTK82

The fact that preprochymosin was not expressed efficiently during heat shock suggested that the patterns of expression observed for NP were specific to it, and neither due to different rates of transcription during heat shock, nor simply a function of the method of introduction of the DNA. However, it was still possible that the variability in expression was a result of the great variation in levels of expression observed between individual oocytes, combined with the relatively small sample size - for practical reasons, there were often less than 10 oocytes per sample - rather than to real differences in translatability of the RNA under different conditions.

It was definitively demonstrated that NP and preprochymosin are expressed differently by coinjecting the two DNAs into the same oocytes (fig. VII.5). In this experiment, the preprochymosin RNA was, as expected, translated efficiently only at 20°C (tracks 5-8). NP RNA was, however, preferentially translated during heat shock (tracks 1-4): although its translation was reduced slightly during heat shock, it was translated much more efficiently than preprochymosin. Obviously if the nucleus of any oocyte had been 'missed' it would contain neither DNA, whereas if it had been 'hit' it would contain both. As the two constructs contained identical promoters, the rates of transcription should have been similarly affected by heat shock. Therefore, the difference in the pattern of expression of the two proteins must have been due to post-transcriptional differences in the behaviour of the RNA.

VII.E Discussion

Why can RNA transcribed when NP genes are injected into oocytes be translated preferentially during heat shock, when chymosin RNA introduced in the same way cannot? NP RNA is not endogenous to *Xenopus*, so it is difficult to believe that the mechanism is specific to NP. It is much more likely that the RNA happens to contain features allowing it to be recognized by the mechanisms responsible for preferential translation of endogenous hsp messages. Comparison of the DNA sequences of *Xenopus* hsp70 and influenza NP (Bienz, 1984b; Huddleston and Brownlee, 1982) does reveal a region of striking homology, present at roughly the same position in the untranslated leaders of the hsp70 and pTKNP RNAs:



The pTKNP RNA leader also contains TK sequences, so in natural NP mRNA this sequence is in fact only 15 nucleotides downstream of the cap. Interestingly, it is also present at the same position in the 5' leaders of all influenza mRNAs (Lamb and Choppin, 1983). It is not present in *Drosophila* hsp70 mRNA, which is not preferentially translated in oocytes (Bienz and Pelham, 1982).

It was tempting to conclude that the presence of these similar sequences in the otherwise unrelated mRNA species might explain their similar translational behaviour, although the evidence for this was purely circumstantial. It therefore seemed worthwhile to attempt to assess the importance of this

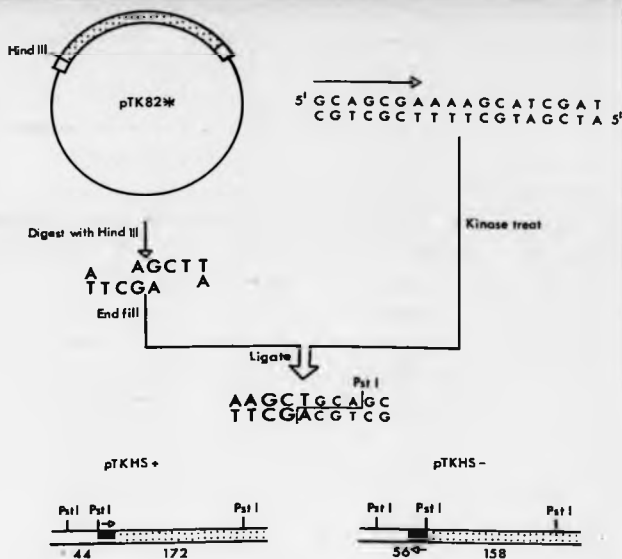


FIG. VII.6 CONSTRUCTION OF pTKH5+ AND pTKH5-.

After linearization with HindIII, pTK82+ was end filled using Klenow enzyme and CIP treated. The 18-mers were treated with kinase, then ligated with the vector. The 4 bases at the 'left hand' end of the oligonucleotide form a PstI site when ligated with an end-filled HindIII site. It was therefore possible to determine the orientation of insertion by digesting the resulting constructs with PstI; this generated a 172-bp fragment from pTK82+ and a 158-bp fragment from pTK82-.

homologous sequence, as a possible short cut to identifying the sequences involved in preferential translation. This was done by inserting a double-stranded oligonucleotide containing the hsp70 version of the sequence into another gene, and testing whether RNA transcribed on injection of the modified gene into oocytes was translated during heat shock.

VII.F Insertion of heat shock oligonucleotide into pTK82 and expression in oocytes

VII.F.1. Insertion of oligonucleotide

The obvious candidate for the modification procedure described above was pTK82: it was already known that its RNA was predominantly translated at 20°C in oocytes, and the oligos could be inserted into the middle of the 5' leader at the HindIII site joining the TK and preprochymosin sequences. This operation was made even simpler by using pTK82*, identical to pTK82, except that it had lost the HindIII site at the 3' end of the preprochymosin insert during the subcloning (Strachan, 1986). Although it was not known how many bases had been lost, in oocytes this construct gave rise to RNA which was efficiently translated to yield bona fide preprochymosin (R. Strachan, unpublished data).

The DNA was linearised with HindIII, end filled and ligated with the oligo (fig. VII.6). In addition to the hsp70 homologous sequence, the oligo also contained a ClaI site and 4 bases of a PstI site. This was included so that if insertion occurred faithfully, with no loss of bases from either fragment, a new, diagnostic site would be formed with the end-filled HindIII site (see fig. VII.6). Initial screening of transformants was therefore by digestion with these enzymes.

Because PstI cut the DNA only at one end of the oligonucleotide, the orientation of insertion could also be assessed from the sizes of fragments generated by PstI digestion: if the consensus sequence was inserted in the same orientation as in hsp70 and NP, a 172bp fragment was generated; if it was in the opposite orientation, a 158bp fragment was generated (fig. VII.6).

Two transformants were selected that contained the oligonucleotides in opposite orientations, termed pTK82HS+ and pTK82HS-. Orientation was confirmed by subcloning a 658 bp EcoRI fragment spanning the insertion site into M13mp18, and sequencing (data not shown).

VII.F.ii Expression

An attempt was made to express the three constructs pTK82HS+, pTK82HS-, and pTK82* in oocytes by co-injecting each with pTKNP, allowing transcription to occur, and radioactively labelling the oocytes at either 20°C or 35°C. However, no expression of preprochymosin was observed in any of the samples, incubated at either temperature. NP was expressed, but in this experiment it was not preferentially translated during heat shock (data not shown, but the pattern of expression was the same as shown in fig. VII.2c). Therefore, even if preprochymosin had been synthesized, these oocytes would not necessarily have been expected to translate the modified RNA during heat shock.

This result was frustrating as time did not permit the experiment to be repeated. It illustrates the problem inherent in the design of the experiment: if the oligo has an effect, it may only be seen in oocytes which can preferentially

translate NP RNA, but there is no way of predicting in advance which oocytes can do this.

VII.G Translation of injected RNA

As has already been discussed (see VI.A), interpretation of the results of experiments studying translational control during heat shock would be greatly simplified if RNA, rather than DNA, could be injected. Since natural mRNA for the proteins discussed in this chapter was readily available, this approach had the additional advantage that, for initial experiments at least, the difficulties encountered with hsp70 in synthesizing in vitro RNA with all the features needed for translation in vivo would be avoided. Influenza virus-infected cells contain so much NP mRNA that simply selecting poly(A)+ RNA produces a preparation in which it is one of the major species. The preparation also contains the other flu RNAs, and these are of particular interest because their 5' leaders all share the 'consensus sequence' with NP, and so might be predicted to share the same translational behaviour. Other cell types are similarly rich sources of natural ovalbumin and preprochymosin mRNA.

In a series of experiments, these natural mRNAs were injected into oocytes, and their translation monitored at normal temperatures and during heat shock. As predicted, calf abomasum preprochymosin mRNA and hen oviduct ovalbumin mRNA were translated much more efficiently at 20°C than at 35°C (fig. VII.7a tracks 7-9, and VII.7c tracks 1-4). This was obviously not a result of degradation of RNA during heat shock, as the rate of translation of ovalbumin increased slightly during the second heat shock, and dramatically during

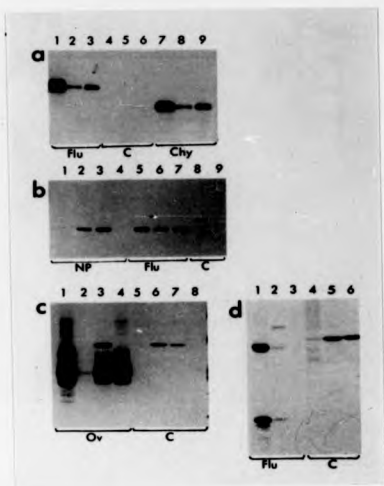


FIG. VII.7 TRANSLATION OF INJECTED mRNA IN OOCYTES DURING HEAT SHOCK.

In all experiments, RNA was injected at 2ng per oocyte, and DNA was injected at 4ng per oocyte. After overnight incubation at 20°C, the oocytes were labelled for 2h in MBS containing 1mCi/ml [³⁵S]methionine, under some or all of the following conditions: 20°C; at 35°C; during a second heat shock (after a 2h period at 20°C); at 20°C, during the second 2h period of recovery after a heat shock. Surviving oocytes were analysed by immunoprecipitation with the appropriate antibody, and electrophoresis on 12.5% SDS-polyacrylamide gels.

FIG. VII.7a TRANSLATION OF PREPROCHYMOSIN AND NP mRNAs. Tracks 1-3, preprochymosin and flu mRNA, monoclonal anti-NP (S/1); 4-6, uninjected, monoclonal anti-NP and rabbit anti-prochymosin; 7-9, preprochymosin and flu mRNA, rabbit anti-prochymosin. Oocytes incubated at 20°C (tracks 1, 4, and 7), or during the first (2, 5, and 8), or second (3, 6, and 9) heat shock; 0.75 oocyte per track.

FIG. VII.7b TRANSLATION OF pTKNP RNA AND NATURAL NP mRNA. Tracks 1-4, pTKNP DNA; 5-7, flu mRNA; 8 and 9, uninjected. Oocytes incubated at 20°C (tracks 1, 5, and 8), during the first (2, 6, and 9) or second (3 and 7) heat shocks, or during recovery (4); monoclonal anti-NP used for all samples; 0.75 oocyte per track.

FIG. VII.7c TRANSLATION OF OVIDUCT POLY(A)+ RNA. Tracks 1-4, oviduct poly(A)+ RNA; 5-8, uninjected control oocytes. Oocytes were labelled at 20°C (tracks 1 and 5), during the first (2 and 6) or second (3 and 7) heat shocks, or during recovery (4 and 8); rabbit anti-lysozyme used for all samples; 1.25 oocyte loaded per track.

FIG. VII.7d TRANSLATION OF FLU mRNA. Tracks 1-3, flu mRNA; 4-6, uninjected controls. Oocytes labelled at 20°C (tracks 1 and 4), or during the first (2 and 5) or second (3 and 6) heat shocks; rabbit anti-flu antibody used for all immunoprecipitations; 0.75 oocytes per track.

recovery, in line with the overall level of protein synthesis.

Surprisingly, though, translation of NP poly(A)+ RNA from influenza-infected cells also occurred predominantly at 20°C (fig. VII.7a, tracks 1-3, and fig. VII.7b), even in experiments in which RNA transcribed from pTKNP was preferentially translated at 35°C, and regardless of the type of response mounted by the oocytes, and of the concentration of RNA injected (data not shown). Translation of the other influenza mRNAs was also reduced by heat shock (fig. VII.7d).

There are two possible reasons for this difference in behaviour. The first is that RNA injected directly into the cytoplasm is not handled by oocytes in the same way as RNA transcribed from genes injected into the germinal vesicle, so that the sequences controlling preferential translation are not 'read' correctly by the translational apparatus. The second is that it is due to the slight differences in structure between natural flu NP message and the RNA transcribed from pTKNP, which has 56 bases of the thymidine kinase 5' leader at its 5' end. It would be difficult to distinguish these possibilities experimentally.

Chapter VIII. CONCLUSIONS AND PROSPECTS

VIII.A Introduction

When this project was begun, it was thought that Xenopus laevis oocytes responded to increased temperature by beginning to translate previously synthesized 'masked' hsp70 mRNA (Biens and Gurdon, 1982). The original aim was therefore to identify regions of this mRNA that allow it to be masked and unmasked and to be selectively translated during heat shock. However, the doubts raised early in the project about the nature of the heat shock response (King and Davis, 1987) changed its emphasis, and the response itself was investigated thoroughly before the original aim was pursued.

Consequently, the conclusions drawn fall into three related categories: (1) the nature of the heat shock response mounted by Xenopus laevis oocytes and the follicle cells attached to them; (2) the effects of heat shock on the translation of exogenous transcripts encoding hsp70 and other proteins; (3) more general observations on the use of oocytes as a transcription/translation system, and in particular as a system to study signals involved in translational control.

The conclusions in all these categories raise further questions, which could be investigated experimentally.

VIII.B The nature of the heat shock response

Conclusions

Oocytes of Xenopus laevis prepared by manual dissection of ovary are each surrounded by approximately 8000 somatic cells, most of which are follicle cells (Chapter III). These cells remain metabolically active during incubation in vitro, contributing up to 50% of total protein synthesis at normal

temperatures, and up to 80% during heat shock. Removal of these cells poses great problems, and a two-step procedure is required to achieve this.

The follicle cells appear to contain no hsp70 mRNA at normal temperatures (around 20°C), but heat shock (incubation at 35°C, in these experiments) induces rapid transcription of hsp70 genes. One oocyte-complement of follicle cells may synthesize up to 125pg hsp70 mRNA in two hours. Translation of 'normal' mRNA molecules is dramatically reduced, but the newly synthesized hsp70 mRNA is efficiently translated. Radiolabelled hsp70 protein is readily detectable on one-dimensional SDS-polyacrylamide gels. The heat shock response mounted by the follicle cells therefore corresponds to the 'classic' somatic cell response.

Importantly, a complete follicle cell layer is not required for these cells to mount a heat shock response: isolated patches of cells remaining after partial defolliculation are capable of making hsp70. After return to normal temperatures, hsp70 mRNA is gradually degraded, and normal patterns of transcription and translation resume.

By contrast, non-heat-shocked oocytes do contain a small amount of hsp70 mRNA (up to 13pg per oocyte, although there is great variation between batches), and this does not increase on heat shock. Surprisingly, although the overall level of protein synthesis did decrease on heat shock, synthesis of hsp70 was never detectable in defolliculated oocytes, regardless of the incubation temperature. This was not because interactions with the follicle cells are necessary for induction of the response, since the results were the same whether the follicle cells were removed before or after

incubation in radioactive medium. Oocytes therefore appear not to respond to heat shock in any way, other than by a reduction in the overall rate of protein synthesis.

Some batches of oocytes mount an unusual response, in which the overall rate of protein synthesis does not decrease, and sometimes even increases, on heat shock. The synthesis of a group of proteins of 60-80 kD is specifically induced in the oocytes. However, none of these corresponds to the hsp70 synthesized in heat-shocked follicle cells. There is circumstantial evidence that this response is related to an environmental factor, rather than to genetic differences between frogs.

These results indicate that all hsp70 attributed to oocytes in previous studies was in fact synthesized in the follicle cells. Rather than being a remarkable exception, *Xenopus* may be similar to other species (as diverse as *Drosophila* and *Saccharomyces*), in which hsp70 is not inducible during oogenesis or sporulation (Zimmerman et al. 1983; Kurtz et al. 1986). However, these species differ from *Xenopus* oocytes in that hsp70 mRNA has not been detected at these stages.

An alternative explanation might be that, rather than being permanently masked, hsp70 mRNA in *Xenopus* oocytes is translated some or all of the time, but at levels too low to be detected by the methods used in this study.

Prospects

In some batches of oocytes there is one tenth as much hsp70 mRNA as in an oocyte-complement of heat-shocked follicle cells. If translated at the same efficiency as that in follicle cells, this would be expected to produce easily detectable amounts of hsp70 protein. So, if it is translated in oocytes, then either (1) the rate of translation of hsp70 mRNA or the proportion of the molecules in polysomes must be lower than in follicle cells, or (2) the concentration of free methionine must be much higher. The former possibility would not be unexpected, since general translation is suppressed in oocytes (see Taylor *et al.* 1985a). It is also possible that the original hypothesis of Bienz and Gurdon is in fact correct - albeit that the levels of hsp70 involved must be much lower than they suggested.

These alternatives should be resolvable by analysis of sucrose density gradients of defolliculated oocytes incubated at 20°C or at 35°C, to reveal whether the hsp70 mRNA is in the polysomal or non-polysomal fractions and whether there is any change in distribution on heat shock. A problem with this approach is that the amount of hsp70 mRNA is very variable, and sometimes almost undetectable. As discussed in III.G, it was attempted once, but was unsuccessful because the levels of hsp70 mRNA in that batch were very low.

VIII.C Effects of heat shock on translation of exogenous transcripts

Conclusions

Plasmids containing Xenopus hsp70A genes (and their promoters) are efficiently transcribed in oocytes at normal temperatures, yielding up to 4ng per oocyte of RNA of which the length and polyadenylation status are indistinguishable from the RNA induced in follicle cells by heat shock (Chapter IV). When oocytes are incubated in labelled medium both at normal temperatures and during heat shock, this RNA produces easily detectable amounts of hsp70 protein. During heat shock, hsp70 RNA is efficiently translated although translation of other RNAs is greatly reduced (Chapter V). In some experiments, the rate of hsp70 synthesis actually increases on heat shock (when the overall rate of protein synthesis decreases).

In other words: (1) oocytes can translate hsp70 mRNA at 20°C; and (2) during heat shock its translation is preferential compared with other mRNA species. However, the amount of hsp70 produced is comparable to that made in follicle cells, although the amount of RNA present may be ten times as great. The rate of synthesis of radioactive hsp70 is therefore much lower than in follicle cells - an effect which may be related to the method by which the transcripts are introduced into oocytes, or, as discussed above, to either the general suppression of protein synthesis in oocytes or differences in methionine pool size.

DNA encoding influenza virus nucleoprotein under control of the HSV TK promoter is also efficiently transcribed in oocytes (Chapter VII). In many (but not all) batches of

oocytes, this RNA behaves in the same way as hsp70 RNA. That is, it is translated preferentially during heat shock. Occasionally it is apparently 'masked', i.e. translated detectably only during heat shock. In contrast preprochymosin RNA transcribed under control of the same promoter is always translated more efficiently at normal temperatures than during heat shock. The behaviour of NP RNA therefore appears to be specific, and related to its sequence rather than to the fact that it is transcribed from injected genes. [However, when natural flu mRNA was injected directly, preferential translation was never observed (see VIII.D)].

Prospects

The preferential translation of hsp70 RNA in oocytes suggests that the approach of injecting hsp70 genes with mutations in sequences corresponding to the 5' leader would yield information about the signals involved in this phenomenon. The next step would be to create a series of plasmids with different short deletions or insertions in that region and analyse their expression. It would obviously be very advantageous to alter the coding region so that the product could be distinguished from native hsp70 without analysis on two-dimensional gels. However, the difficulties encountered in detecting translation products from a fusion gene and a truncated gene even when RNA was injected directly into oocyte cytoplasm (to eliminate problems of transport of hsp70B-derived transcripts out of the nucleus) suggest that modifications to hsp70 tend to make it unstable.

The behaviour of TKNP RNA and the presence of a region in its leader sequence highly homologous with Xenopus hsp70 makes it tempting to speculate that this sequence is the signal required for preferential translation during heat shock. It was unfortunate that time did not permit thorough analysis of the plasmid containing this sequence in the preprochymosin gene, which was designed to test this. Since it might provide a short cut to identifying a control region, it would be profitable to inject this plasmid into oocytes again, and follow the synthesis of preprochymosin at normal and heat-shock temperatures - in a batch of oocytes that preferentially translates NP RNA.

Little is known about the signals involved in translational control in general. They may well involve the secondary structure of the message, in which case interactions between regions of RNA widely separated in the linear sequence might be important, and short sequences might not act autonomously as signals when transferred to a different environment. However, although based on a rather simplistic hypothesis, this type of experiment is worthwhile since there is as yet no evidence to disprove it. The heat shock response of Xenopus oocytes provides a suitable model system.

VIII.D Oocytes as a system for investigating translational control

Conclusions

To investigate translational control, it is necessary that the same amount of RNA is present in the cytoplasm under all conditions of the experiment. When hsp70A genes are injected, this is true. However, heat shock does induce a

slight increase in transcription, although all the new transcripts remain in the nucleus. When hsp70B genes are injected, few transcripts are transported to the cytoplasm even at normal temperatures, and the induction of transcription on heat shock is much more marked. These features make hsp70B an unsuitable candidate for leader-sequence mutagenesis. In general therefore, it is clearly important to assay transcription under all conditions to be used in the experiments before investigating translation.

To attempt to circumvent these problems, hsp70 RNA synthesized *in vitro* was injected (Chapter VI). Like RNA transcribed from injected genes, this was also translated efficiently - if it contained sequences resembling a poly(A) tail at its 3' end. Even molecules containing a short open reading frame upstream of the correct translation initiation site were translated, and removing the upstream AUG did not significantly affect the rate of translation. However, on heat shock the rate of translation of all synthetic RNA was reduced in parallel with that of endogenous non-heat-shock mRNAs. It thus appears that injected RNA is not subject to the same translational regulation as RNA transcribed in the oocyte, even if from injected genes. It is possible that translational control mechanisms cannot act on the large amounts of previously synthesized RNA injected. For example, binding of protein factors or sequence modification may require nascent or newly synthesized RNA.

Results with influenza RNA supported this conclusion (Chapter VII). It was never translated preferentially in oocytes during heat shock; translation was always reduced to

the same extent as other exogenous RNAs such as ovalbumin and globin. However, the recent report that, when synthetic polyadenylated RNA encoding ribosomal protein L1 is injected into oocytes, it is subject to the same regulation during maturation as is the corresponding endogenous mRNA (Hyman and Wormington, 1988), indicates that this method is viable, at least for some RNAs or some mechanisms of translational control.

Prospects

In the cases tested, the RNA injected into oocytes was not identical with the corresponding RNA transcribed in oocytes: for NP, the injected RNA was the 'natural' RNA and the RNA transcribed from pTKNP had additional sequences at its extreme 3' and 5' ends; for hsp70, the RNA transcribed in oocytes was 'natural', and that transcribed in vitro had additional sequences at the ends of the molecule. It is therefore possible that the results were due not to differences in the method of introducing the RNA, but to the slight differences in structure of RNA introduced by each of the two methods. This problem could be resolved by purifying hsp70 mRNA from heat shocked frog tissue and injecting this into oocytes.

IX. REFERENCES

- Amin, J., Mestrl, R., Schiller, P., Dreano, M., and Voellmy, R. (1987). Organization of the Drosophila melanogaster hsp70 heat shock regulation unit. Mol. Cell. Biol. 7, 1055-1062.
- Anderson, C. W., Straus, J. W., and Dudoock, B. S. (1983). Preparation of a cell-free protein-synthesizing system from wheat germ. Methods Enzymol. 101, 635-644.
- Anderson, D. M., Richter, J. D., Chamberlain, M. E., Price, D. H., Britten, R. J., Smith, L. D., and Davidson, E. H. (1982). Sequence organization of poly(A) RNA synthesized and accumulated in lamprush chromosome stage Xenopus laevis oocytes. J. Mol. Biol. 155, 281-309.
- Angerer, L. M., and Angerer, R. C. (1981). Detection of poly(A)+ RNA in sea urchin eggs and embryos by quantitative in situ hybridization. Nucleic Acids Res. 9, 2819-2840.
- Angerer, L. M., DeLeon, D. V., Angerer, R. C., Showman, R. M., Wells, D. E., and Raff, R. A. (1984). Delayed accumulation of maternal histone mRNA during sea urchin oogenesis. Dev. Biol. 101, 477-484.
- Arrigo, A-P. (1987). Cellular localization of hsp23 during Drosophila development and following subsequent heat shock. Dev. Biol. 122, 38-48.
- Arrigo, A-P., and Ahmad-Zadeh, C. (1981). Immunofluorescence localization of a small heat shock protein (hsp23) in salivary glands and cells of Drosophila melanogaster. Mol. Gen. Genet. 184, 73-79.
- Arrigo, A-P., Darlix, J-L., Khandjian, E. W., Simon, M., and Spahr, P-F. (1985). Characterization of the prosome from Drosophila and its similarity to the cytoplasmic structures formed by the low molecular weight heat-shock proteins. EMBO J. 4, 399-408.
- Ashburner, M., and Bonner, J. J. (1979). The induction of gene activity in Drosophila by heat shock. Cell 17, 241-254.
- Audet, R. G., Goodchild, J., and Richter, J. D. (1987). Eukaryotic initiation factor 4A stimulates translation in microinjected oocytes. Dev. Biol. 121, 58-68.
- Ballantine, J. E. M., Woodland, H. R., and Sturgess, E. A. (1979). Changes in protein synthesis during the development of Xenopus laevis. J. Embryol. Exp. Morphol. 51, 137-153.
- Ballinger, D. G., and Hunt, T. (1981). Fertilization of sea urchin eggs is accompanied by 40S ribosomal subunit phosphorylation. Dev. Biol. 87, 277-285.

Ballinger, D. G., and Pardue, M-L. (1983). The control of protein synthesis during heat shock in Drosophila cells involves altered polypeptide initiation rates. Cell **33**, 103-114.

Ballinger, D., and Pardue, M-L. (1985). Mechanism of translational control in heat-shocked Drosophila cells. In Changes in Eukaryotic Gene Expression in Response to Environmental Stress. (London: Academic Press), pp. 349-371.

Ballinger, D. G., Bray, S. J., and Hunt, T. (1984). Studies of the kinetics and ionic requirements for the phosphorylation of ribosomal protein S6 after fertilization of Arhacia punctulata eggs. Dev Biol. **101**, 192-200.

Barnier, J. V., Bensaude, O., Morange, M., and Babinet, C. (1987). Mouse 89kD heat shock protein: two polypeptides with distinct developmental regulation. Exp. Cell. Res. **170**, 186-194.

Baum, E. Z., and Wormington, W. M. (1985). Coordinate expression of ribosomal protein genes during Xenopus development. Dev. Biol. **111**, 488-498.

Bédard, P-A., and Brandhorst, B.P. (1986). Translational activation of maternal mRNA encoding the heat-shock protein hsp90 during sea urchin embryogenesis. Dev. Biol. **117**, 286-293.

Bensaude, O., Babinet, C., Morange, M., and Jacob, F. (1983). Heat shock proteins: first major products of zygotic gene activity in mouse embryo. Nature **305**, 331-333.

Bienz, M. (1982). The heat shock response in Xenopus oocytes and somatic cells: differences in phenomena and control. In Heat Shock: From Bacteria to Man, M. J. Schlesinger, M. Ashburner, and A. Tissieres, eds. (Cold Spring Harbor Laboratory Press), pp. 177-181.

Bienz, M. (1984a). Developmental control of the heat shock response in Xenopus. Proc. Natl. Acad. Sci. USA **81**, 3138-3142.

Bienz, M. (1984b). Xenopus hsp70 genes are constitutively expressed in injected oocytes. EMBO J. **2**, 2477-2483.

Bienz, M. (1986). A CCAAT box confers cell-type-specific regulation on the Xenopus hsp70 gene in oocytes. Cell **46**, 1037-1042.

Bienz, M., and Gurdon, J. B. (1982). The heat-shock response in Xenopus is controlled at the translational level. Cell **29**, 811-819.

Bienz, M., and Pelham, H. R. B. (1982). Expression of a Drosophila heat-shock protein in Xenopus oocytes: conserved and divergent regulatory signals. EMBO J. **1**, 1583-1588.

Bienz, M., and Pelham, H. R. B. (1986). Heat shock regulatory elements function as an inducible enhancer in the Xenopus hsp70 gene and when linked to a heterologous promoter. Cell **45**, 753-760.

Birnboim, H. C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**, 1513-1523.

Birnstiel, M. L., Busslinger, M., and Strub, K. (1985). Transcription termination and 3' processing: the end is in site! Cell **41**, 349-359.

Blackman, R. K., and Meselson, M. (1986). Interspecific nucleotide sequence comparisons used to identify regulatory and structural features of the Drosophila hsp82 gene. J. Mol. Biol. **188**, 499-515.

Bond, U., and Schlesinger, M. J. (1985). Ubiquitin is a heat shock protein in chicken embryo fibroblasts. Mol. Cell. Biol. **5**, 849-856.

Bonner, W. M., and Laskey, R. A. (1974). A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**, 83-88.

Brachet, J., and Denis, H. (1983). Effects of actinomycin D on morphogenesis. Nature **198**, 205-206.

Browder, L. W., Pollok, M., Heikkila, J. J., Wilkes, J., Wang, T., Krone, P., Ovsenek, O., and Kloc, M. (1987). Decay of the oocyte-type heat shock response of Xenopus laevis. Dev. Biol. **121**, 191-199.

Caldwell, D. C., and Emerson, C. P., Jr (1985). The role of cap methylation in the translational activation of stored maternal histone mRNA in sea urchin embryos. Cell **42**, 691-700.

Capell, T. G., Welch, W. J., Schlossman, D. M., Palter, K. B., Schlesinger, M. J., and Rothman, J. E. (1986). Uncoating ATPase is a member of the 70 kilodalton family of stress proteins. Cell **45**, 3-13.

Chirico, W. J., Waters, M. G., and Blobel, G. (1986). 70k heat shock related proteins stimulate translocation into microsomes. Nature **332**, 805-810.

Chodosh, L. A., Baldwin, A. S., Carthew, R. W., and Sharp, P. A. (1988). Human CCAAT-binding proteins have heterologous subunits. Cell **53**, 11-24.

Clark, B. D., and Brown, I. R. (1986). A retinal heat shock protein is associated with elements of the cytoskeleton and binds to calmodulin. Biochem. Biophys. Res. Commun. **139**, 974-991.

Clemens, M. J. (1984). Translation of eukaryotic messenger RNA in cell-free extracts. In *Transcription and Translation - A Practical Approach*, B. D. Hames and S.J. Higgins, eds. (Oxford: IRL Press), pp. 231-270.

Clemens, M. (1987). Translational control: developments in development. *Nature* 330. 699-700.

Clewell, D. B., and Helsinki, D. R. (1969). Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular form. *Proc. Natl. Acad. Sci. USA* 62, 1159-1166.

Cohen, R. S., and Meselson, M. (1985). Separate regulatory elements for the heat-inducible and ovarian expression of the *Drosophila* hsp26 gene. *Cell* 43, 737-746.

Colbert, R. A., Hucul, J. A., Scorsone, K. A., and Young, D. A. (1987). α Subunit of eukaryotic translational initiation factor-2 is a heat shock protein. *J. Biol. Chem.* 262, 16763-16766.

Colin, A. M., and Hille, M. B. (1986). Injected mRNA does not increase protein synthesis in unfertilized, fertilized, or ammonia-activated sea urchin eggs. *Dev. Biol.* 115, 184-192.

Colin, A. M., Brown, B. D., Dholakia, J. N., Woodley, C. L., Wahba, A. J., and Hille, M. B. (1987). Evidence for simultaneous derepression of messenger RNA and the guanine nucleotide exchange factor in fertilized sea urchin eggs. *Dev. Biol.* 123, 354-363.

Colman, A. (1984a). Expression of exogenous DNA in *Xenopus* oocytes. In *Transcription and Translation - A Practical Approach*, B. D. Hames and S. J. Higgins, eds. (Oxford: IRL Press), pp. 49-69.

Colman, A. (1984b). Translation of eukaryotic messenger RNA in *Xenopus* oocytes. In *Transcription and Translation - A Practical Approach*, B. D. Hames and S. J. Higgins, eds. (Oxford: IRL Press), pp. 271-302.

Corces, V., and Pellicer, A. (1984). Identification of sequences involved in the transcriptional control of a *Drosophila* heat shock gene. *J. Cell. Biol.* 259, 14812-14817.

Craig, E. A., and Jacobsen, K. (1984). Mutations of the heat inducible 70 kilodalton genes of yeast confer temperature sensitive growth. *Cell* 38, 841-849.

Craig, E. A., and Jacobsen, K. (1985). Mutations in cognate genes of *Saccharomyces cerevisiae* hsp70 result in reduced growth rates at low temperatures. *Mol. Cell. Biol.* 5, 3517-3524.

Craig, E. A., Ingolia, T. D., and Manseau, L. J. (1983). Expression of *Drosophila* heat-shock cognate genes during heat shock and development. *Dev. Biol.* 99, 418-426.

- Craig, E. A., Kramer, J., and Kosic-Smithers, J. (1987). SSC1, a member of the 70-kDa heat shock protein multigene family of Saccharomyces cerevisiae is essential for growth. Proc. Natl. Acad. Sci. USA. 84, 4158-4160.
- Curci, A., Bevilacqua, A., and Mangia, F. (1987). Lack of heat-shock response in preovulatory mouse oocytes. Dev. Biol. 123, 154-160.
- Cutler, D. F. (1982). Secretion of chick proteins from Xenopus oocytes. PhD thesis, University of Warwick, pp. 47-49.
- Daniels, C. J., McKee, A. H. Z. and Doolittle, W. F. (1982). Archaeobacterial heat-shock proteins. EMBO J. 1, 745-749.
- Darnbrough, C. H., and Ford, P.J. (1981). Identification in Xenopus laevis of a class of oocyte-specific proteins bound to messenger RNA. Eur. J. Biochem. 113, 415-424.
- Davey, J. (1984). PhD thesis, University of Warwick.
- Davey, J., Colman, A., and Dimmock, N. J. (1985a). Location of influenza virus M, NP, and NS1 proteins in microinjected cells. J. Gen. Virol. 66, 2319-2334.
- Davey, J., Dimmock, N. J., and Colman, A. (1985b). Identification of the sequence responsible for the nuclear accumulation of the influenza virus nucleoprotein in Xenopus oocytes. Cell 40, 687-675.
- Davidson, E. H. (1986). Gene Activity in Early Development (Orlando: Academic Press), 2nd edn.
- Dearsley, A. L., Johnson, R. M., Barrett, P., and Somerville, J. (1985). Identification of a 60-kDa phosphoprotein that binds stored messenger RNA of Xenopus oocytes. Eur. J. Biochem. 150, 95-103.
- DeLeon, D. V., Cox, K. H., Angerer, L. M., and Angerer, R. C. (1983). Most early-variant histone mRNA is contained in the pronucleus of sea urchin eggs. Dev. Biol. 100, 197-206.
- Deshaies, R. J., Koch, B. D., Werner-Washburne, M., Craig, E. A., and Schekman, R. (1988). A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature 332, 800-805.
- DiDomenico, B. J., Bugaisky, G. E., and Lindquist, S. (1982a). Heat shock and recovery are mediated by different translational mechanisms. Proc. Natl. Acad. Sci. USA 79, 6181-6185.
- DiDomenico, B. J., Bugaisky, G. E., and Lindquist, S. (1982b). The heat shock response is self-regulated at both transcriptional and post-transcriptional levels. Cell 31, 593-603.

Dixon, L. K., and Ford, P. J. (1982). Regulation of protein synthesis and accumulation during oogenesis in Xenopus laevis. Dev. Biol. 83, 478-497.

Dolecki, G. J., and Smith, L. D. (1979). Poly(A)+ RNA metabolism during oogenesis in Xenopus laevis. Dev. Biol. 69, 217-236.

Drummond, D. R., Armstrong, J., and Colman, A. (1985). The effect of capping and polyadenylation on the stability, movement and translation of synthetic messenger RNA's in Xenopus oocytes. Nucleic Acids Res. 13, 7375-7394.

Dumont, J. N., and Brummet, A. R. (1978). Oogenesis in Xenopus laevis (Daudin) V. Relationships between developing oocytes and their investing follicular tissues. J. Morphol. 155, 73-88.

Duncan, R., and Hershey, J. W. B. (1984). Heat shock-induced translational alterations in HeLa cells. Initiation factor modifications and the inhibition of translation. J. Biol. Chem. 259, 11882-11889.

Dworkin, M. B., Shrutkowski, A., and Dworkin-Rastl, E. (1985). Mobilization of specific maternal mRNA species into polysomes after fertilization in Xenopus laevis. Proc. Natl. Acad. Sci. 82, 7636-7640.

Evans, T., Rosenthal, E. T., Youngblom, J., Distel, D., and Hunt, T. (1983). Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell 33, 389-398.

Ford, C. C., and Gurdon, J. B. (1977). A method for enucleating oocytes of Xenopus laevis. J. Embryol. Expt. Morphol. 37, 203-209.

Georgiev, O., Mons, J., and Birnstiel, M. L. (1984). Processing and nucleo-cytoplasmic transport of histone gene transcripts. Nucleic Acids Res. 12, 8539-8551.

Giebel, L. B., Dworniczak, B. P., and Bautz, E. K. F. (1988). Developmental regulation of a constitutively expressed mouse mRNA encoding a 72-kDa heat shock-like protein. Dev. Biol. 125, 200-207.

Gil, A., and Proudfoot, N. J. (1987). Position-dependent sequence elements downstream of AAUAAA are required for efficient rabbit β -globin mRNA 3' end formation. Cell 49, 399-408.

Glover, C. V. C. (1982). Heat shock induces rapid dephosphorylation of a ribosomal protein in Drosophila. Proc. Natl. Acad. Sci. USA 79, 1781-1785.

- Golden, L., Schafer, U., and Rosbash, M. (1980). Accumulation of individual β A+ mRNAs during oogenesis of *Xenopus laevis*. *Cell* 22, 835-844.
- Grainger, J. L., and Winkler, M. M. (1987). Fertilization triggers unmasking of maternal mRNAs in sea urchin eggs. *Mol. Cell. Biol.* 7, 3947-3954.
- Green, M. R., Maniatis, T., and Melton, D. A. (1983). Human β -globin pre-mRNA synthesized *in vitro* is accurately spliced in *Xenopus* oocyte nuclei. *Cell* 32, 681-694.
- Guedon, G., Sovia, D., Ebel, J. P., Befort, N., and Remy, P. (1985). Effect of diadenosine tetraphosphate microinjection on heat shock protein synthesis in *Xenopus laevis* oocytes. *EMBO J.* 4, 3743-3748.
- Hames, B. D., and Rickwood, D., eds. (1981). *Gel Electrophoresis of Proteins: A Practical Approach* (Oxford: IRL Press Ltd.).
- Hattori, M., and Sakaki, Y. (1986). Dideoxy sequencing method using denatured plasmid templates. *Anal. Biochem.* 152, 232-238.
- Heikkila, J. J., Miller, J. G. O., Schultz, G. A., Kloc, M., and Browder, L. W. (1985a). Heat shock gene expression during early animal development. In *Changes in Gene Expression in Response to Environmental Stress*, B. G. Atkinson and D. B. Walden, eds. (New York: Academic Press), pp. 135-158.
- Heikkila, J. J., Kloc, M., Bury, J., Schultz, G. A., and Browder, L. W. (1985b). Acquisition of the heat-shock response and thermotolerance during early development of *Xenopus laevis*. *Dev. Biol.* 107, 483-489.
- Hickey, E. D., and Weber, L. A. (1982). Modulation of heat-shock polypeptide synthesis in HeLa cells during hyperthermia and recovery. *Biochemistry* 21, 1513-1521.
- Hinds, P. W., Finlay, C. A., Frey, A. B., and Levine, A. J. (1987). Immunological evidence for the association of p53 with a heat shock protein, hsc70, in p53-plus-ras-transformed cell lines. *Mol. Cell. Biol.* 7, 2863-2869.
- Hiromi, Y., and Hotta, Y. (1985). Actin gene mutations in *Drosophila*: heat shock activation in the indirect flight muscles. *EMBO J.* 4, 1861-1867.
- Horrell, A., Shuttleworth, J., and Colman, A. (1987). Transcript levels and translational control of hsp70 synthesis in *Xenopus* oocytes. *Genes Devel.* 1, 433-444.
- Huang, W-I., Hansen, L. J., Merrick, W. C., and Jagus, R. (1987). Inhibitor of eukaryotic initiation factor 4F activity in unfertilized sea urchin eggs. *Proc. Natl. Acad. Sci. USA* 84, 8359-8363.

Huddleston, J. A., and Brownlee, G. G. (1982). The sequence of the nucleoprotein gene of human influenza A virus strain A/NT/60/68. *Nucleic Acids Res.* **10**, 1029-1038.

Hultin, T. (1981a). The effect of puromycin on protein metabolism and cell division in fertilized sea urchin eggs. *Experientia* **17**, 410-411.

Hultin, T. (1981b). Activation of ribosomes in sea urchin eggs in response to fertilization. *Exp. Cell Res.* **25**, 405-425.

Hultmark, D., Klemenz, R., and Gehring, W. J. (1986). Translational and transcription control elements in the untranslated leader of the heat-shock gene hsp22. *Cell* **44**, 429-438.

Hunt, C., and Morimoto, R. I. (1985). Conserved features of eukaryotic hsp70 genes revealed by comparison with the nucleotide sequence of human hsp70. *Proc. Natl. Acad. Sci. USA* **82**, 6455-6459.

Hunt, T., Hunter, T., and Munro, A. (1969). Control of haemoglobin synthesis: rate of translation of the mRNA for the α - and β -chains. *J. Mol. Biol.* **43**, 123-134.

Hyman, L. E., and Wormington, W. M. (1988). Translational inactivation of ribosomal protein mRNAs during *Xenopus* oocyte maturation. *Genes Devel.* (in press).

Iida, H., and Yahara, I. (1985). Yeast heat-shock protein of Mr48000 is an isoprotein of enolase. *Nature* **315**, 688-690.

Ilan, J., and Ilan, J. (1978). Translation of maternal messenger ribonucleoprotein particles from sea urchin in a cell-free system from unfertilized eggs and product analysis. *Dev. Biol.* **66**, 375-385.

Ingolia, T. D., and Craig, E. A. (1982). Four small *Drosophila* heat shock proteins are related to each other and to mammalian α -crystallin. *Proc. Natl. Acad. Sci. USA* **79**, 2360-2364.

Jackson, R. J. (1986). The heat shock response in *Drosophila* KCI61 cells. *Eur. J. Biochem.* **152**, 623-634.

Jackson, R. J., and Hunt, T. (1983). Preparation and use of nuclease-treated rabbit reticulocyte lysates for the translation of eukaryotic messenger RNA. *Methods Enzymol.* **96**, 51-74.

Jenkins, N. A., Kaumeyer, J. E., Young, E. M., and Raff, R. A. (1978). A test for masked message: the template activity of messenger ribonucleoprotein particles isolated from sea urchin eggs. *Dev. Biol.* **63**, 279-298.

Kaufman, R. J., Murtha, P., and Davies, M. V. (1987). Translational efficiency of polycistronic mRNAs and their utilization to express heterologous genes in mammalian cells. *EMBO J.* **6**, 187-193.

Kay, R. J., Boissy, R. J., Russnak, R. H., and Candido, E. P. M. (1986). Efficient transcription of a Caenorhabditis elegans heat shock gene pair in mouse fibroblasts is dependent on multiple promoter elements which can function bidirectionally. Mol. Cell. Biol. **6**, 3134-3143.

Kessler, S. W. (1975). Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of the antibody-antigen complexes with protein A. J. Immunol. **115**, 1617-1624.

Kick, D., Barrett, P., Cummings, A., and Sommerville, J. (1987). Phosphorylation of a 60kDa polypeptide from Xenopus blocks messenger RNA translation. Nucleic Acids Res. **11**, 4099-4109.

King, M-L., and Davis, R. (1987). Do Xenopus oocytes have a heat shock response? Dev. Biol. **119**, 532-539.

Klemenz, R., and Gehring, W. J. (1986). Sequence requirement for expression of the Drosophila melanogaster heat shock protein hsp22 gene during heat shock and normal development. Mol. Cell. Biol. **6**, 2011-2019.

Klemenz, R., Hultmark, D., and Gehring, W. J. (1985). Selective translation of heat shock mRNA in Drosophila melanogaster depends on sequence information in the leader. EMBO J. **4**, 2053-2060.

Klotzel, P-M., and Bautz, E. K. F. (1983). Heat-shock proteins are associated with hnRNA in Drosophila melanogaster tissue culture cells. EMBO J. **2**, 705-710.

Korneluk, R. G., Quan, F., and Gravel, R. A. (1985). Rapid and reliable dideoxy sequencing of double-stranded DNA. Gene **40**, 317-323.

Kozak, M. (1984a). Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. Nucleic Acids Res. **12**, 857-872.

Kozak, M. (1984b). Selection of initiation sites by eukaryotic ribosomes: effect of inserting AUG triplets upstream from the coding sequence for preproinsulin. Nucleic Acids Res. **12**, 3873-3893.

Kozak, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44**, 283-293.

Kozak, M. (1987). At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. J. Mol. Biol. **196**, 947-950.

Kramer, B., Kramer, W., and Fritz, H-J. (1984). Different base/base mismatches are corrected with different efficiencies by the methyl-directed DNA mismatch-repair system of E. coli. Cell **38**, 879-887.

Kressman, A., Clarkson, S. G., Pirotta, V., and Birnstiel, M. L. (1978). Transcription of cloned tRNA gene fragments and subfragments injected into the oocyte nucleus of Xenopus laevis. Proc. Natl. Acad. Sci. USA 75, 1176-1180.

Krieg, P. A., and Melton, D. A. (1984). Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. Nucleic Acids Res. 112, 7057-7070.

Krieg, P. A., and Melton, D. A. (1987). Methods Enzymol. (in press).

Krieg, P., Strachan, R., Wallis, E., Tabe, L., and Colman, A. (1984). Efficient expression of cloned complementary DNAs for secretory proteins after injection into Xenopus oocytes. J. Mol. Biol. 180, 615-643.

Kruger, C., and Benecke, B. (1981). In vitro translation of Drosophila heat shock and non-heat shock mRNAs in heterologous and homologous cell-free systems. Cell 23, 593-603.

Kurtz, S., Rossi, J., Petko, L., and Lindquist, S. (1986). An ancient developmental induction: heat-shock proteins induced in sporulation and oogenesis. Science 231, 1154-1157.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lai, B-T., Chin, N. W., Stanek, A. E., Keh, W., and Lanks, K. W. (1984). Quantitation and intracellular localization of the 85K heat shock protein by using monoclonal and polyclonal antibodies. Mol. Cell. Biol. 4, 2802-2810.

Lamb, R. A., and Chopin, P. W. (1983). The gene structure and replication of influenza virus. Annu. Rev. Biochem. 52, 467-506.

Lanks, K. W. (1986). Modulators of the eukaryotic heat shock response. Exp. Cell. Res. 165 1-10.

Laskey, R. A., Mills, A. D., Gurdon, J. B., and Partington, G. A. (1977). Protein synthesis in oocytes of Xenopus laevis is not regulated by the supply of messenger RNA. Cell, 11, 345-351.

Lee, G., Hynes, R., and Kirschner, M. (1984). Temporal and spatial regulation of fibronectin in early Xenopus development. Cell 36, 729-740.

Lewis, M. J., and Pelham, H. R. B. (1985). Involvement of ATP in the nuclear and nucleolar functions of the 70 kD heat shock protein. EMBO J. 4, 3137-3143.

Lindquist, S. (1980a). Varying patterns of protein synthesis in Drosophila during heat shock: implications for regulation. Dev. Biol. 77, 463-479.

- Lindquist, S. (1980b). Translational efficiency of heat-induced messages in Drosophila melanogaster cells. *J. Mol. Biol.* **137**, 151-158.
- Lindquist, S. (1981). Regulation of protein synthesis during heat shock. *Nature* **293**, 311-314.
- Lindquist, S. (1985). Heat shock response: difference means to the same end. In *Microbiology-1985*, L. Leive, and D. Schlessinger, eds. (Washington: American Society for Microbiology), pp. 332-335.
- Lindquist, S. (1986). The heat-shock response. *Annu. Rev. Biochem.* **55**, 1151-1191.
- Lindquist, S., and McGarry, T. J. (1986). Translational control of heat shock proteins in Drosophila. In *Translational Control*, M. B. Mathews, ed. (Cold Spring Harbour Laboratory Press), pp. 88-90.
- Lingrel, J. B., and Woodland, H. R. (1974). Initiation does not limit the rate of globin synthesis in message-injected Xenopus oocytes. *Eur. J. Biochem.* **47**, 47-56.
- Loomis, W. F., and Wheeler, S. (1980). Heat shock response of Dictyostelium. *Dev. Biol.* **79**, 399-408.
- Lorenz, L. J., and Richter, J. D. (1985). A cDNA clone for a polyadenylated RNA-binding protein of Xenopus laevis oocytes hybridizes to four developmentally regulated mRNAs. *Mol. Cell. Biol.* **5**, 2697-2704.
- Maniatis, T., Fritsch, E. F., and Sambrook, J., eds. (1982). *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbour Laboratory Press).
- Manrow, R. E., and Jacobson, A. (1987). Increased rates of decay and reduced levels of accumulation of the major poly(A)-associated proteins of Dictyostelium during heat shock and development. *Proc. Natl. Acad. Sci. USA* **84**, 1858-1862.
- McGarry, T. J., and Lindquist, S. (1985). The preferential translation of Drosophila hsp70 mRNA requires sequences in the untranslated leader. *Cell* **42**, 903-911.
- McKenzie S. L., Henikoff, S., and Meselson, M. (1975). Localization of RNA from heat-induced polysomes at puff sites in Drosophila melanogaster. *Proc. Natl. Acad. Sci. USA* **72**, 1117-1121.
- McMahon, A. P., Novak, T. J., Britten, R. J., and Davidson, E. H. (1984). Inducible expression of a cloned heat shock fusion gene in sea urchin embryos. *Proc. Natl. Acad. Sci. USA* **81**, 7490-7494.

- Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., and Green, M. R. (1984). Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* **12**, 7035-7358.
- Mitchell, H. K., Petersen, N. S., and Buzin, C. H. (1985). Self degradation of heat shock proteins. *Proc. Natl. Acad. Sci. USA* **82**, 4968-4973.
- Moon, R. T., Moe, K. D., and Hille, M. B. (1980). Polypeptides of nonpolyribosomal messenger ribonucleoprotein complexes of sea urchin eggs. *Biochemistry* **19**, 2723-2730.
- Moon, R. T., Danilchik, M. V., and Hille, M. B. (1982). An assessment of the masked message hypothesis: sea urchin egg messenger ribonucleoprotein complexes are efficient templates for in vitro protein synthesis. *Dev. Biol.* **93**, 389-403.
- Moor, R. M., and Osborn, J. C. (1983). Somatic control of protein synthesis in mammalian oocytes during maturation. *Ciba Foundation Symp.* **88**, 178-196.
- Munro, S., and Pelham, H. R. B. (1984). Use of peptide tagging to detect proteins expressed from cloned genes: deletion mapping functional domains of Drosophila hsp70. *EMBO J.* **3**, 3087-3093.
- Munro, S. and Pelham, H. R. B. (1985). What turns on heat shock genes? *Nature* **317**, 477-478.
- Nakamaye, K. I., and Eckstein, F. (1986). Inhibition of restriction endonuclease NciI cleavage by phosphothioate groups and its application to oligonucleotide-directed mutagenesis. *Nucleic Acids Res.* **14**, 9679-9698.
- Neidhardt, F. C., VanBogelen, R. A., and Vaughn, V. (1984). The genetics and regulation of heat-shock proteins. *Annu. Rev. Genet.* **18**, 295-329.
- Newport, J., and Kirschner, M. (1982). A major developmental transition in early Xenopus embryos: I. Characterization and timing of cellular changes at the midblastula stage. *Cell* **30**, 675-686.
- Nickells, R. W., and Browder, L. W. (1985). Region specific heat-shock protein synthesis correlates with a biphasic acquisition of thermotolerance in Xenopus laevis embryos. *Dev. Biol.* **112**, 391-395.
- O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007-4021.

Old, R. W., and Primrose, S. B. (1985). Principles of Gene Manipulation, 3rd edn. (Oxford: Blackwell Scientific Publications).

Olsen, A. S., Treimer, D. F., and Sanders, M. M. (1983). Dephosphorylation of S6 and expression of the heat shock response in Drosophila melanogaster. Mol. Cell. Biol. 3, 2017-2027.

Operman, H., Levinson, W., and Bishop, J. M. (1981). A cellular protein that associates with the transforming protein of Rous sarcoma virus is also a heat-shock protein. Proc. Natl. Acad. Sci. USA 78, 1067-1071.

Palatnik, C. M., Wilkins, C., and Jacobson, A. (1984). Translational control during early Dictyostelium development: possible involvement of poly(A) sequences. Cell 36, 1017-1025.

Parker, C. S., and Topol, J. (1984). A Drosophila RNA polymerase II transcription factor binds to the regulatory site of an hsp70 gene. Cell 37, 273-283.

Pelham, H. R. B. (1982). A regulatory upstream promoter element in the Drosophila hsp70 heat-shock gene. Cell 30, 517-528.

✓ Pelham, H. R. B. (1986). Speculations on the functions of the major heat shock and glucose-regulated proteins. Cell 46, 959-961.

Pelham, H. (1988). Heat-shock proteins: coming in from the cold. Nature, 332, 776-777.

Pelham, H. R. B., and Bienz, M. (1982). Synthetic heat-shock promoter element confers heat-inducibility on the herpes simplex virus thymidine kinase gene. EMBO J. 1, 1473-1477.

Pelham, H., Lewis, M., and Lindquist, S. (1984). Expression of a Drosophila heat shock protein in mammalian cells: transient association with nucleoli after heat shock. Phil. Trans. R. Soc. London, Ser. B 307, 301-307.

Pierandrei-Amaldi, P., Campioni, N., Beccari, E., Bozzoni, I., and Amaldi, F. (1982). Expression of ribosomal protein genes in Xenopus laevis development. Cell 30, 163-171.

Plesofsky-Vig, N., and Brambl, R. (1985). Heat shock response of Neurospora crassa: protein synthesis and induced thermotolerance. J. Bacteriol. 162, 1083-1091.

Richter, J. D., and Smith, L. D. (1983). Developmentally regulated RNA binding proteins during oogenesis in Xenopus laevis. J. Cell. Biol. 258, 4864-4869.

Richter, J. D., and Smith, L. D. (1984). Reversible inhibition of translation by Xenopus oocyte-specific proteins. Nature 309, 378-380.

Richter, J. D., Wasserman, W. J., and Smith, L. D. (1982). The mechanism for increased protein synthesis during Xenopus oocyte maturation. *Dev. Biol.* **82**, 159-167.

Richter, J. D., Smith, L. D., Anderson, D. M., and Davidson, E. H. (1984). Interspersed poly(A) RNAs of amphibian oocytes are not translatable. *J. Mol. Biol.* **173**, 227-241.

Riddihough, G., and Pelham, H. R. B. (1986). Activation of the Drosophila hsp27 promoter by heat shock and ecdysterone involves independent and remote regulatory sequences. *EMBO J.* **5**, 1653-1658.

Riddihough, G., and Pelham, H. R. B. (1987). An ecdysone responsive element in the Drosophila hsp27 promoter. *EMBO J.* **6**, 3729-3734.

Rigby, P. W. J., Dieckman, M., Rhodes, C., and Berg, P. (1977). Labelling DNA to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* **113**, 237-251.

Roccheri, M. C., di Bernado, M. G., and Giudice, G. (1981). Synthesis of heat-shock proteins in developing sea urchins. *Dev. Biol.* **83**, 173-177.

Roccheri, M. C., Sconzo, G., LaRossa, M., Oliva, D., Abrigniani, A., and Giudice, G. (1986). Response to heat shock of different sea urchin species. *Cell. Diff.* **18**, 131-135.

Rose, D. W., Wettenhall, R. E. H., Kudicki, W., Kramer, G., Hardesty, B. (1987). The 90-kilodalton peptide of the heme-regulated eIF-2 α kinase has sequence similarity with the 90-kilodalton heat shock protein. *Biochemistry* **26**, 2583-2587.

Rosenthal, E. T., and Ruderman, J. V. (1987). Widespread changes in the translation and adenylation of maternal messenger RNAs following fertilization of Spisula oocytes. *Dev. Biol.* **121**, 237-248.

Rosenthal, E. T., Hunt, T., and Ruderman, J. V. (1980). Selective translation of mRNA controls the pattern of protein synthesis during early development of the surf clam, Spisula solidissima. *Cell* **20**, 487-494.

Rosenthal, E. T., Tansey, T. R., and Ruderman, J. V. (1983). Sequence-specific adenylations and deadenylations accompany changes in the translation of maternal messenger RNA after fertilization of Spisula oocytes. *J. Mol. Biol.* **166**, 309-327.

Sakmann, B., Methfessel, C., Mishina, M., Takahashi, T., Takai, T., Kurasaki, M., Fukuda, K., and Numa, S. (1985). Role of acetyl choline receptor subunits in gating of the channel. *Nature* **318**, 538-543.

Saunders, M. M. (1981). Identification of histone H2B as a heat-shock protein in Drosophila. *J. Cell Biol.* **91**, 579-583.

✓ Schlesinger, M. J., Ashburner, M., and Tissieres, A. eds. (1982). Heat Shock: From Bacteria to Man (Cold Spring Harbour Laboratory Press).

Scorsone, G., Roccheri, M. C., La Rossa, M., Oliva, D., Abrignani, A., and Giudice, G. (1986). Acquisition of thermotolerance in sea urchin embryos correlates with the synthesis and age of heat shock proteins. *Cell Diff.* **12**, 173-177.

Scorsone, K. A., Panniers, R., Rowlands, A. G., and Henshaw, E. C. (1987). Phosphorylation of eukaryotic initiation factor-2 during physiological stresses which affect protein synthesis. *J. Biol. Chem.* **262**, 14538-14543.

Scott, M. P., and Pardue, M-L. (1981). Translational control in lysates of *Drosophila melanogaster* cells. *Proc. Natl. Acad. Sci. USA* **78**, 3353-3357.

Shih, R., O'Connor, C., Keem, K., and Smith, L. D. (1978). Kinetic analysis of amino acid pools and protein synthesis in amphibian oocytes and embryos. *Dev. Biol.* **68**, 172-182.

Showman, R. M., Wells, D. E., Anstrom, J., Hursh, D. A., and Raff, R. A. (1982). Message-specific sequestration of maternal histone mRNA in the sea urchin egg. *Proc. Natl. Acad. USA* **79**, 5944-5947.

Showman, R. M., Leaf, D. S., Anstrom, J. A., and Raff, R. A. (1987). Translation of maternal histone mRNAs in sea urchin embryos: a test of control by cap methylation. *Dev. Biol.* **121**, 284-287.

Sincox, A. M., Cheney, C. M., Hoffman, E. P., and Shearn, A. (1985). A deletion of the 3' end of the *Drosophila melanogaster* hsp70 gene increases stability of mutant mRNA during recovery from heat shock. *Mol. Cell. Biol.* **5**, 3397-3402.

Slater, A., Cato, A. C. B., Sillar, G. M., Kioussis, J., and Burdon, R. H. (1981). The pattern of protein synthesis induced by heat shock of HeLa cells. *Eur. J. Biochem.* **117**, 341-346.

Smith, L. D., and Richter, J. D. (1985). Synthesis, accumulation and utilization of maternal macromolecules during oogenesis. In *Biology of Fertilization*, C. Metz, and A. Monroy, eds. (New York: Academic Press), pp. 141-188.

Spena, A., Hain, R., Ziervogel, U., Saedler, H., and Schell, J. (1985). Construction of a heat-inducible gene for plants. Demonstration of a heat-inducible activity of the *Drosophila* hsp70 promoter in plants. *EMBO J.* **4**, 2739-2743.

Standart, N. M., Bray, S. J., George, E. L., and Hunt, T. (1985). The small subunit of ribonucleotide reductase is encoded by one of the most abundant translationally regulated maternal mRNAs in clam and sea urchin eggs. *J. Cell Biol.* **100**, 1968-1976.

Standart, N., Hunt, T., and Ruderman, J. V. (1986). Differential accumulation of ribonucleotide reductase subunits in clam oocytes: the large subunit is stored as a polypeptide, the small subunit as untranslated mRNA. *J. Cell Biol.* 103, 2129-2136.

Standart, N., Minshall, J., Pines, J., and Hunt, T. (1987). Cyclin synthesis, modification and destruction during meiotic maturation of the starfish oocyte. *Dev. Biol.* 124, 248-258.

Stick, R., and Hausen, P. (1985). Changes in the nuclear lamina composition during early development of *Xenopus laevis*. *Cell* 41, 191-200.

Stith, B. J., and Maller, J. L. (1985). Increased intracellular pH is not necessary for ribosomal protein S6 phosphorylation, increased protein synthesis, or germinal vesicle breakdown in *Xenopus* oocytes. *Dev. Biol.* 107, 460-469.

Storti, R. V., Scott, M. P., Rich, A., and Pardue, M-L. (1980). Translational control of protein synthesis in response to heat shock in *D. melanogaster* cells. *Cell* 22, 825-834.

Strachan, F. R. (1986). The autonomy and function of eukaryotic signal sequences: Segregation of variants of preprochymosin and prelysozyme in *Xenopus* oocytes and *in vitro*. PhD thesis, University of Warwick, pp 67-72.

Swenson, K. I., Borgese, N., Pietrini, G., and Ruderman, J. V. (1987). Three translationally regulated mRNAs are stored in the cytoplasm of clam oocytes. *Dev. Biol.* 123, 10-16.

Tabe, L., Kreig, P., Strachan, R., Jackson, D., Wallis, E., and Colman, A. (1984). Segregation of mutant ovalbumins and ovalbumin-globin fusion proteins in *Xenopus* oocytes: identification of an ovalbumin signal sequence. *J. Mol. Biol.* 180, 645-666.

Tas, P. W. L., and Martini, O. H. W. (1987). Regulation of ribosomal protein S6 phosphorylation in heat-shocked HeLa cells. *Eur. J. Biochem.* 163, 553-559.

Taylor, M. A., and Smith, L. D. (1985). Quantitative changes in protein synthesis during oogenesis. *Dev. Biol.* 110, 230-237.

Taylor, M. A., Johnson, A. D., and Smith, L. D. (1985a). Growing *Xenopus* oocytes have spare translational capacity. *Proc. Natl. Acad. Sci. USA* 82, 6586-6589.

Taylor, M. A., Robinson, K. R., and Smith, L. D. (1985b). Intracellular pH and ribosomal protein S6 phosphorylation: role in translational control in *Xenopus* oocytes. *J. Embryol. Exp. Morphol.* 82 (Suppl.) 35-51.

Thomas, P. S. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA* 77, 5201-5205.

Torok, I., and Karch, F. (1980). Nucleotide sequences of heat shock activated genes in *Drosophila melanogaster*. I. Sequences in the regions of the 5' and 3' ends of the hsp70 gene in the hybrid plasmid 56H8. *Nucleic Acids Res.* **8**, 3105-3123.

Ungewickell, E. (1985). The 70 kD mammalian heat shock proteins are structurally and functionally related to the uncoating protein that releases clathrin triskelia from coated vesicles. *EMBO J.* **4**, 3385-3391.

Venezky, D. L., Angerer, L. M., and Angerer, R. C. (1981). Accumulation of histone repeat transcripts in the sea urchin egg pronucleus. *Cell* **24**, 385-391.

Voellmy, R., and Rungger, D. (1982). Transcription of a *Drosophila* heat shock gene is heat-induced in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **79**, 1776-1780.

Ward, G. E., Vacquier, V. D., and Michel, S. (1983). The increased phosphorylation of ribosomal protein S6 in *Arbacia punctulata* is not a universal event in the activation of sea urchin eggs. *Dev. Biol.* **95**, 360-371.

Wasserman, W. J., Richter, J. D., and Smith, L. D. (1982). Protein synthesis during maturation promoting factor- and progesterone-induced maturation in *Xenopus* oocytes. *Dev. Biol.* **89**, 152-158.

Welch, W. J., and Feramisco, J. R. (1984). Nuclear and nucleolar localization of the 72,000-dalton heat shock protein in heat-shocked mammalian cells. *J. Biol. Chem.* **259**, 4501-4513.

Werner-Washburne, M., Stone, D. E., and Craig, E. A. (1987). Complex interactions among members of an essential subfamily of hsp70 genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **7**, 2568-2577.

Williams, J. G., Kay, R. M., and Patient, R. K. (1980). The nucleotide sequence of major β -globin mRNA from *Xenopus laevis*. *Nucleic Acids Res.* **8**, 4247-4256.

Wilt, F. H. (1977). The dynamics of maternal poly(A)-containing mRNA in fertilized sea urchin eggs. *Cell* **11**, 673-681.

Winkler, M. M., Nelson, E. M., Lashbrook, C., and Hershey, J. W. B. (1985). Multiple levels of regulation of protein synthesis at fertilization in sea urchin eggs. *Dev. Biol.* **107**, 290-300.

Winkler, M. M., Grainger, J. L., and Kelso, L. C. (1986). Masked mRNA and protein synthesis in the sea urchin egg. *J. Cell Biol.* **103** (Suppl.) 85a.

Woodland, H. R. (1974). Changes in the polysome content of developing *Xenopus laevis* embryos. *Dev. Biol.* **40**, 90-101.

Woodland, H. R. (1980). Histone synthesis during the development of Xenopus. FEBS Lett. 121, 1-7.

Wu, B. J., Kingston, R. E., and Morimoto, R. I. (1986). Human hsp70 promoter contains at least two distinct regulatory domains. Proc. Natl. Acad. Sci. USA 83, 829-833.

Wu, B. J., Williams, G. T., and Morimoto, R. I. (1987). Detection of three protein binding sites in the serum-regulated promoter of the human gene coding for the 70-kDa heat shock protein. Proc. Natl. Acad. Sci. USA 84, 2203-2207.

Zimmerman, J. L., Petri, W., and Meselson, M. (1983). Accumulation of a specific subset of D. melanogaster heat shock mRNAs in normal development without heat shock. Cell 32, 1161-1170.

Zinn, K., DiMaio, D., and Maniatis, T. (1983). Identification of two distinct regulatory regions adjacent to the human β -interferon gene. Cell 34, 865-879.

Zoller, M. J., and Smith, M. (1983). Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol. 100, 468-500.

APPENDIX

Transcript levels and translational control of hsp70 synthesis in *Xenopus* oocytes

Ann Horrell, John Shuttleworth, and Alan Colman

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

Transcript levels and translational control of hsp70 synthesis in *Xenopus* oocytes

Ann Horrell, John Shuttleworth, and Alan Colman

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

Until recently it was believed that *Xenopus* oocytes respond to heat shock by synthesizing the 70-kD heat shock protein hsp70 and that, uniquely amongst animal cell types, this response is mediated entirely at the translational level. This view has now been challenged and we present data that reevaluate the involvement of translational control in the heat shock response of *Xenopus* oocytes. RNase mapping shows that up to 13 pg of hsp70A and hsp70B mRNA are accumulated by fully grown oocytes in the absence of heat shock. These transcripts are retained stably during maturation, fertilization, early cleavage, and following heat shock. However, no hsp70 protein synthesis can be detected by two-dimensional polyacrylamide gel analysis of [³⁵S]methionine-labeled proteins from completely defolliculated oocytes, either before or during heat shock. Oocytes injected with hsp70A DNA rapidly accumulate high levels of hsp70 mRNA in their cytoplasm at normal temperature. During heat shock these oocytes accumulate more transcripts, but they remain in the nucleus and cytoplasmic levels remain constant. Translation of hsp70 from these transcripts is readily detectable at non-heat shock and heat shock temperatures. We conclude that (1) "exogenous" hsp70 transcripts are efficiently translated and not masked at normal temperatures in oocytes, and (2) oocytes are able to selectively translate hsp70 mRNA during heat shock.

[Key words: *Xenopus*; hsp70; mRNA levels; translational control]

Received April 4, 1987; revised version accepted May 7, 1987

The ability to produce heat shock proteins (hsp) in response to increased temperature (together with a variety of other physiological stresses) is a property common to most, if not all, eukaryotic cells [Schleinger et al. 1982, Pelham, 1985]. Heat shock-induced expression of hsp has been well characterized, notably in *Drosophila* where regulation occurs at both the transcriptional and translational levels [Di Domenico et al. 1982, Hultmark et al. 1986]. Induction involves the transient and coordinate activation of heat shock genes, through the binding of the heat shock transcription factor (HSTF) to highly conserved heat shock sequence elements (HSEs) in their promoter regions [Pelham 1982, Parker and Topol 1984]. Rapidly accumulated hsp mRNA is then selectively translated at the elevated temperature while translation of most other cellular mRNAs is repressed [Storti et al. 1980]. The mechanisms underlying this translational selectivity remain unclear but are thought to involve (1) alterations in the efficiency of the translational apparatus of heat shocked cells and (2) sequences present in the 5' untranslated leader of hsp mRNA that facilitate preferential translation [Clover 1982, McGarry and Lindquist 1985, Hultmark et al. 1986].

Developmental and noncoordinate regulation of hsp gene expression has also been reported in a variety of nonstress cells from *Drosophila*, yeast, human, and

mouse [Zimmerman et al. 1983, Kurtz et al. 1986, Wu and Morimoto 1986, Bensaude et al. 1983]. The presence of independent regulatory sequences, distinct from HSEs, in the promoter regions of several of these genes is providing some insight into their expression during normal cell growth and differentiation [Riddibough and Pelham 1986, Wu and Morimoto 1986]. Recent studies on the developmental control of heat shock gene expression in *Xenopus* have indicated that alternative transcriptional and translational mechanisms may be utilized in oocytes. The hsp70 genes are thought to be transcribed constitutively during oogenesis controlled by the activity of both HSE and CCAAT box regulatory elements in their promoters [Bienz 1984b, 1986]. Significant amounts of hsp70 mRNA are accumulated but do not appear to be translated until the oocytes are heat shocked [Bienz and Curdon 1982, Bienz 1984a]. hsp70 synthesis by heat-shocked oocytes has therefore been attributed to translational activation followed by preferential translation of preexisting, masked hsp70 mRNA. In contrast, hsp70 transcripts and hsp70 synthesis have not been detected in unfertilized eggs or early embryos, facts that appear to correlate with their lack of thermotolerance [Bienz 1984a, Heikkilä et al. 1985]. Together these results imply that hsp70 transcripts in oocytes contain features allowing them, first, to be masked at normal

temperatures, second, to be preferentially translated during heat shock, and, third, to be selectively destroyed during maturation.

We became interested in *hap70* expression during *Xenopus* oogenesis since it appeared to represent a novel example of translational control. However, recent evidence has suggested that the data on translation of *hap70* by heat-shocked oocytes may be complicated by a contribution of protein derived from the several thousand follicle cells that initially surround each oocyte in the ovary. King and Davis [1987] could not detect *hap70* protein synthesis in defolliculated oocytes, and question the presence of *hap70* mRNA in stage VI oocytes. To reinvestigate the phenomenon of translational activation of *hap70* mRNA we have used methods that ensure the complete removal of all follicle cells from the oocyte. Our initial studies have reevaluated the accumulation, stability, and fate of *hap70* mRNA in oocytes, eggs, and embryos. Using a sensitive RNase protection assay, we find that maternal *hap70A* and *hap70B* transcripts accumulate early in oogenesis. These transcripts are stable during heat shock in stage VI oocytes and, contrary to previous reports, persist in undiminished amounts in eggs and in stage 2, 8, and 11 embryos.

Using one- and two-dimensional polyacrylamide gel electrophoresis, we also fail to detect any *hap70* protein synthesis from the transcripts in defolliculated stage VI oocytes either before or during heat shock. This could be because the rate of *hap70* protein synthesis from endogenous transcripts is below the limit of detection of our analysis. We have therefore investigated an alternative strategy of expressing *hap70* protein from injected genes. We present here data on the accumulation and localization of *hap70A* mRNA and its translation in oocytes. Translation of *hap70* protein is readily detectable at non-heat shock and heat shock temperatures in DNA-injected oocytes. These results will allow the design of experiments to investigate the structural features of *hap70* mRNA that facilitate preferential translation during heat shock.

Results and discussion

Defolliculation procedure

Xenopus oocytes are surrounded by two layers of somatic cells—an outer thecal layer containing blood vessels and fibroblasts, and an inner layer which on a stage VI oocyte [Dumont 1972] contains about 6000 ± 1000 follicle cells [see Materials and methods]. To determine the relative contribution of *hap70* RNA and protein from the oocytes and these somatic cells (which we refer to simply as follicle cells), it was necessary to devise a method of completely defolliculating oocytes. We have tested a variety of different methods, but find that the following procedure is the quickest and most effective. Oocytes are given a brief collagenase treatment before removal of the thecal layer by manual dissection. After a second collagenase treatment, the oocytes are incubated in a hypertonic medium [see Materials and methods] in which the vitelline envelope becomes separated from the plasma membrane. This is then removed manually, together with the follicle cells embedded in it.

Removal of the somatic cells can be monitored by staining with Hoechst dye followed by visualization with a fluorescent microscope. This technique has the advantage that it does not require the sample to be fixed, so RNA and protein synthesis can be analyzed in the stained oocytes themselves, rather than in a parallel sample. Control experiments (not shown) show that the various procedures do not affect the recovery of radiolabeled proteins. Figure 1a shows an untreated (or folliculated) oocyte. The dark band in the center of the picture is a blood vessel. Nuclei in the thecal layer have a distinctive comma shape and are more sparsely distributed than those in the inner follicle layer. Removing the theca also removes variable numbers of cells from the inner layer (Fig. 1b). The remainder are embedded in the vitelline membrane and invariably removed with it (Fig. 1c). Therefore, it is crucial to remove both the theca and



Figure 1. Oocytes at various stages of defolliculation, stained with Hoechst dye, and photographed under UV and visible light: (a) a folliculated oocyte, (b) an oocyte with islands of follicle cells remaining after removal of the theca, and (c) a completely defolliculated oocyte. Scale bar, 10 μ m.

the vitelline membrane to ensure that no follicle cells are left attached to the oocyte.

Oogenic hsp70 mRNA levels

The presence of significant amounts of hsp70 mRNA in non-heat-shocked *Xenopus* oocytes is central to the proposition that hsp70 expression is mediated at the translational level, by mRNA recruitment or activation, in these cells. *Xenopus laevis* is known to possess several different hsp70 genes but their relative levels of expression in oocytes is not known. The complete (hsp70A) and partial (hsp70B) sequence information that has been published for two of the genes shows considerable homology in the 5' untranslated regions of the mRNA [Bienz 1984b]. We have obtained additional sequence data for the hsp70B gene, extending from the AhoII site - 16 upstream of the cap site to the HindIII site at +471 in the coding region. Figure 2 shows that in addition to the single base changes already reported at positions -13, -1, 18, 21, and 46 the hsp70B leader sequence contains seven additional bases at positions 102 to 108 when compared with the published hsp70A sequence. This region of the hsp70B gene has been cloned into pGEM-1 and used to transcribe in vitro a 506-nucleotide anti-sense RNA probe using SP6 RNA polymerase [13 nucleotides from the AhoII site to the cap site, 476 nucleotides from the cap site to the end of the HindIII site, and 17 nucleotides of vector sequence]. When the probe is annealed to hsp70B mRNA and digested with RNase, a 476-nucleotide protected fragment is generated [see Fig. 2]. However, with hsp70A mRNA a shorter 368-nucleotide protected fragment is formed because of the deletion in its leader [see Fig. 2]. This probe can therefore detect correctly initiated hsp70B transcripts and also distinguish the relative proportion of hsp70A and hsp70B transcripts in an RNase protection assay.

The relative levels of hsp70 mRNA present in stage VI oocytes, with and without attached follicle cells, both before and after a 2-hr heat shock at 35°C, are shown in Figure 3. Non-heat-shocked oocytes clearly contain detectable levels of both hsp70A and hsp70B mRNA [Fig. 3, lanes 1 and 3]. The level of transcripts is identical in both folliculated and defolliculated oocytes at 20°C indicating that they are contained within the oocyte. The amount of hsp70 mRNA present has been estimated by titrating various amounts of synthetic sense hsp70 mRNA [from 2.5 to 5000 pg] in the RNase protection assay and comparing the counts eluted from the gel for each sample [data not shown]. Up to 6×10^4 copies [10 pg] of hsp70A and 2×10^4 copies [3 pg] of hsp70B mRNA are present per oocyte. The values are similar to the 3×10^4 copies per oocyte quoted by Bienz [1984a]. However, it should be noted that, curiously, we have found that both the absolute and relative amounts of hsp70A and hsp70B mRNA vary considerably between frogs for reasons which are not known [data not shown]. In most experiments levels around 5- to 10-fold less are obtained, while in different frogs the oocyte expression of hsp70A mRNA may be greater or less than hsp70B

mRNA, an effect that is invariably paralleled in the accompanying follicle cells during heat shock.

Heat shock results in no detectable increase in hsp70 mRNA within the oocyte [Fig. 3, lanes 3 and 4] while there is a dramatic increase in hsp70 mRNA attributable to induction of follicle cell genes [Fig. 3, lanes 1 and 2]. Up to 4.2×10^7 hsp70A and 3.4×10^7 hsp70B transcripts are synthesized per oocyte complement of follicle cells, nearly 10-fold the level of transcripts present within the oocyte. This represents a transcription rate of 7×10^3 to 8×10^3 transcripts/gene per hour, assuming 6000 follicle cells/oocytes [see above] and, since *Xenopus laevis* is tetraploid, four hsp70A and four hsp70B genes/nucleus. Additional confirmation of the identity of the protected fragments in Figure 3 was obtained by independently injecting either hsp70A or hsp70B DNA into oocyte nuclei. The transcripts accumulated by injected oocytes were mapped and found to give rise to protected fragments identical in size to those indicated in Figure 3 [data not shown].

In addition to hsp70A- and hsp70B-derived protected fragments, there is a further band of around 80 nucleotides in length omitted from Figure 3 but indicated by c in Figure 4. This RNase-protected fragment is derived from an mRNA present in defolliculated oocytes at 20°C whose levels are not affected by heat shock. This band is most likely derived from heat shock cognate gene transcripts [Craig et al. 1983] present within the oocyte. These are known to have considerable homology to hsp70 and are readily detected as a smaller 2.4-kb band separate from the heat-inducible 2.6-kb hsp70 mRNA on Northern blots of oocyte RNA using an hsp70B probe [data not shown].

The stability of oocyte hsp70 transcripts

The data shown in Figure 3 also demonstrate that hsp70 transcripts are not lost from the oocyte during the course of a single heat shock. If the heat shock-induced activation of hsp70 mRNA is reversible and repeatable within 48 hr [Bienz and Curdon 1982], this would require reutilization of existing transcripts and poses a key question relating to the stability of oocyte hsp70 mRNA. During a conventional heat shock response, mediated at the transcriptional level, hsp transcripts are degraded during recovery from heat shock provided that sufficient functional hsp has accumulated [Di Domenico et al. 1982]. The data in Figure 3, lanes 2 and 5, show that this behavior is demonstrated by the transcripts induced in the follicle cells, where 70% disappear in the 24-hr recovery period. In contrast when oocytes are allowed to recover for 24 hr at 20°C following a single heat shock, the oocyte levels of hsp70 mRNA are unaltered [Fig. 3, lanes 4 and 7]. This latter observation does not take into account any contribution from transcription occurring after the time at which heat shock was reversed, but this would amount to very little when considering the oocyte's potential for replacing hsp70 transcripts during only a 24-hr period. This implies that if translation of hsp70 by oocytes ceases after recovery


```

708 -1 6      5'-----3'
TTTGGGCGATGTTGGTATGACAGATCTTAAAGCATCTTTGAGATCTGACAGAGAG
708 -1 6      TTTCAGGCGATGTTGGGATGACAGAGATCTAAAGCATCTTTGAGATCTGACAGAGAG
      5'-----3'
45      AACAACTGCACAGCGATCTTACTTGGGCAAGAGCGAGCTGGCATATCTTACGAGAAAG
45      AGCAACTGCACAGCGATCTTACTTGGGCAAGAGCGAGCTGGCATATCTTACGAGAA
      ***
105      CBTGAACAAACTGTAAAGGAGCGCAATATGTCACCAACAAAGGAGTCCGAGTCCGCAATGA
102      ***
      AAGCAAACTGTAAAGGAGCGCAATATGTCACCAACAAAGGAGTCCGAGTCCGCAATGA
      (5'-----3')
145      CCTCGGCACACTACTACTCTTGGCTTGGTGTTTTCCACATGGTAAAGTAAAGATCATCC
158      CCTCGGCACACTACTACTCTTGGCTTGGTGTTTTCCACATGGTAAAGTAAAGATCATCC
225      TAAATGACCAAGGGAATCGACCACTCCCACTATGTGGCTTTCACACACAGAAAGGCT
218      TAAATGACCAAGGGAATCGACCACTCCCACTATGTGGCTTTCACACACAGAAAGGCT
285      GATTGGCGATGCAGCAAGAAATCAAGTGGCCATGACCCCAAGATACAGTGTTTGATGC
279      GATTGGCGATGCAGCAAGAAATCAAGTGGCCATGACCCCAAGATACAGTGTTTGATGC
345      CAGAGAGATGATCGGAGAAAGTTCATGATCCCTGATGACATGATGATGATGATGATG
338      CAGAGAGATGATCGGAGAAAGTTCATGATCCCTGATGACATGATGATGATGATGATG
405      GCGCTTCCAAATGGTGAACGATGAGGGAAGGCTTAAGGTCAAGTGGAGTATAAAGGAG
398      GCGCTTCCAAATGGTGAACGATGAGGGAAGGCTTAAGGTCAAGTGGAGTATAAAGGAG
445      B-----3'
      AGGAAAGGCTT
458      A-----3'
      AGGAAAGGCTT

```

Figure 2. The 5' leader sequences of hap70A and hap70B mRNA. The 484-bp *AhaIII*-*HindIII* fragment from the hap70B subclone pXL16P (Bienz 1984b) was inserted into M13, mp18, and mp19 and sequenced by dideoxynucleotide chain termination. The hap70B sequence we obtained is compared with the published hap70A sequence (Bienz 1984b). Mismatches are indicated by an asterisk (*). The hap70A cap site, presumed hap70B cap site, and first ATG codon for each sequence are underlined. The same *AhaIII*-*HindIII* fragment was subcloned in pGEM-1 and transcribed *in vitro* using SP6 RNA polymerase to produce a high-specific activity anti-sense RNA probe. The 5' and 3' limits of probe protection are indicated for hap70B transcripts [---B---] and hap70A transcripts [---A---].

from heat shock, then existing hsp70 transcripts must be translationally repressed, as originally suggested by Bienz and Gurdon (1982).

The oogenic accumulation and fate of hsp70 transcripts

It has already been suggested that to accumulate several million transcripts in a stage VI oocyte, all hap70 genes must be transcribed efficiently throughout most of oogenesis (Bienz and Gurdon 1982). This would also require hap70 mRNA to be highly stable in the oocyte. By contrast the loss of hsp70 mRNA reported to occur during maturation (Bienz and Gurdon 1982) would necessitate destabilization of hsp70 mRNA. In view of the wide variation in oocyte hap70 mRNA levels (see above) and to examine these opposing effects on stability,

hap70 transcripts have been assayed in Dumont stages I-VI oocytes, unfertilized eggs, and stages 2, 8, and 11 embryos. Maximum hap70 mRNA levels are accumulated by around stage III (see Fig. 4a). This suggests that either a steady-state rate of transcription and decay is reached at stage III or that the genes are no longer transcribed after stage III. Unfortunately, these alternatives are not readily distinguished experimentally. Colden et al. (1980) have reported a similar but not identical pattern of accumulation for several nonmitochondrial mRNAs, maximum levels being reached before vitellogenesis (i.e., during stage II).

Taking a figure of 6×10^4 transcripts/oocyte and a rate of 500-1000 transcripts/gene per hour, (see above), an oocyte would require up to 62 days to accumulate its

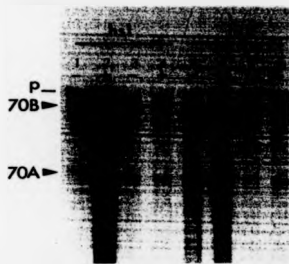


Figure 3. The stability of hsp70 transcripts following heat shock. The relative levels of hsp70A and hsp70B transcripts (indicated by arrow) were analyzed in folliculated (lanes 1 and 2) and defolliculated (lanes 3 and 4) oocytes both before (-) and after (+) a single heat shock at 35°C for 2 hr (lanes 5 and 6). Heat shocked oocytes were allowed to recover for 24 hr at 20°C and then the same analysis was performed on folliculated (lanes 5 and 6) and defolliculated (lanes 7 and 8) oocytes before (-) and after (+) a second heat shock at 35°C for 2 hr (lanes 9 and 10). The RNA equivalent to one oocyte was analyzed in each case. Residual full-length probe is indicated [p].

hsp70 transcripts. There appears to be little agreement in the literature about the kinetics of oogenesis. However, a recent study by Callen et al. [1980] suggests a period of 2 years for production of mature oocytes in the first wave of oogenesis, stage III being achieved between 7 and 10 months after metamorphosis. This is clearly more than sufficient time to allow the accumulation of hsp70 mRNA. However, oogenesis is a highly discontinuous process and the ovary of a mature female contains oocytes arrested at various stages. A large proportion of the oocytes are arrested in late stage I for several years. The age of a frog may then be a factor influencing the variation in hsp70 mRNA levels between frogs if transcription is initiated at metamorphosis. The fact that stage I oocytes removed from a newly metamorphosed frog contain no detectable hsp70 transcripts (data not shown) whereas equivalent-sized oocytes from a mature frog do [Fig. 4a] supports this notion. Continued accumulation of poly(A)⁺ during vitellogenesis and variability in the duration of oogenesis have previously been suggested to explain large variations in mRNA content of fully grown oocytes [Cabada et al. 1977; Darnbrough and Ford 1979].

We have found that, contrary to previous reports, unfertilized eggs and two-cell, midblastula, and midgastrula embryos contain as much hsp70 mRNA as stage VI oocytes that were obtained from the same frog (Fig. 4b). It would appear, therefore, that maternal hsp70 transcripts persist throughout early embryogenesis and no profound changes in stability occur during maturation or

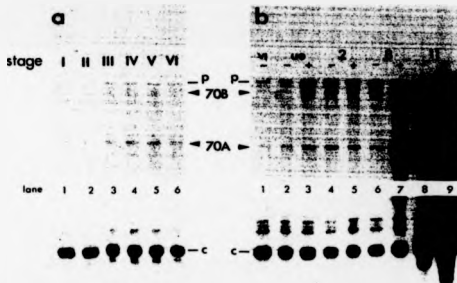


Figure 4. Oogenic accumulation and fate of hsp70 transcripts. [a] Total RNA was extracted from non heat-shocked, nondefolliculated stage I-VI oocytes (lanes 1-6) and assayed for hsp70 transcripts by RNase protection as in Fig. 2. The upper panel shows the relative levels of hsp70A and hsp70B transcripts (indicated by arrows) at each stage. Total RNA equivalent to five oocytes (~22.5 µg) was used in each case. For comparison, the lower panel shows the relative levels of presumptive heat shock cognate transcripts (c) from the same gel. Residual full-length probe is indicated [p]. [b] Total RNA was extracted from stage VI oocytes (VI), unfertilized eggs (ue) and stages 2, 8, and 11 embryos (2, 8, and 11, respectively) before (-) and after (+) a 2-hr heat shock at 35°C. The relative amounts of hsp70A and hsp70B transcripts were assayed as in a using total RNA equivalent to five oocytes, eggs, or embryos in each case.

fertilization. The unfertilized eggs and stage 2 embryos did not survive the 2-hr heat shock at 33°C. The reasons for lack of thermotolerance in eggs and early embryos therefore cannot include loss of hap70 mRNA per se, although alterations in its translatability or capacity for translational activation remain possible. Heat-induced transcripts from embryonic genes are detectable at midblastula and their quantity is proportionately increased in relation to cell number by midgastrula (Fig. 4b, lanes 7 and 9).

hap70 protein synthesis in oocytes and follicle cells

The patterns of protein synthesis we observe in heat-shocked oocytes fall into two distinct categories, which appear to correspond to the type of heat shock response described by Bienz and Gurdon (1982) and that described by Guedon et al. (1985).

In about 70% of batches of oocytes, the pattern of protein synthesis in folliculated oocytes closely resembles that described by Bienz and Gurdon (1982). Figure 5 shows representative examples of protein profiles of folliculated oocytes labeled with [³⁵S]methionine at 20°C and 35°C on both one dimensional (Fig. 5a, tracks 1 and 3) and two-dimensional gels (Figs. 5b, c). On heat shock

the overall rate of protein synthesis falls dramatically (by up to 90%, data not shown), and hap70 (marked H) is the major protein synthesized. At 20°C, hap70 synthesis is not detectable, but two other proteins of molecular weight 70,000 are made (Fig. 5b, proteins 1 and 2). Their mobilities are so similar to that of hap70 that it seems likely that they are the related heat shock cognates (Craig et al. 1983). Surprisingly, the translation of these two proteins appears in this experiment to be maintained at the higher temperature (Fig. 5, cf. panels b and c). However this selective translation is not always observed (Fig. 6, cf. panels b and d).

To answer the question of whether any hap70 is made in the oocytes themselves, oocytes were defolliculated after labeling (Fig. 5a, tracks 2 and 4). Clearly the follicle cells make a significant contribution to overall protein synthesis since there is less radioactive protein in the defolliculated than in the folliculated samples. Estimates based on acid precipitation of incorporated [³⁵S]methionine from oocyte homogenates are that the follicle cells can contribute up to about 50% of the overall protein synthesis at 20°C, and up to 80% at 35°C (data not shown). Most importantly for these investigations, though, all of the labeled hap70 protein is removed

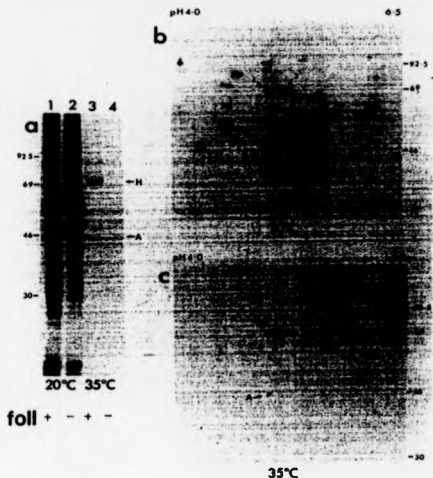


Figure 5. Normal pattern of heat shock protein synthesis. Oocytes complete with follicle cells were incubated in medium containing [³⁵S]methionine for 3 hr at 20°C (i.e., tracks 1 and 2) or at 35°C (tracks 3 and 4) and analyzed on 10% SDS polyacrylamide gels before (tracks 1 and 3) and after (tracks 2 and 4) defolliculation. Folliculated oocytes incubated in label at 20°C (b) or 35°C (c) were also analyzed by two-dimensional electrophoresis. Sizes of molecular weight markers are in kilodaltons. Proteins marked 1 and 2 are discussed in the text. [H] hap70.

with the follicle cells (Fig. 5a, track 4). No *hap70* protein can be detected on two-dimensional gels from oocytes defolliculated after incubation at 20°C or 35°C (data not shown).

Moor and Osborn (1983) have demonstrated that the removal of follicle cells from mammalian oocytes results in a dramatic and specific reduction in actin synthesis within the oocyte, thus indicating that signals from the follicle cells can regulate protein synthesis within the oocyte. It was therefore a possibility (albeit remote) that the failure of the oocyte to translate its store of heat shock mRNA was in some way related to its intimate relationship with the surrounding follicular layer. In the experiment presented in Figure 6 we examined the effects of the removal of follicle cells prior to heat shock on the synthesis of both actin and *hap70*. Clearly, although there is a generally lower incorporation of [³⁵S]methionine into protein in defolliculated oocytes, there is no specific reduction in actin synthesis at 20°C. Evidently the mammalian situation has no counterpart in amphibians. In addition, no *hap70* synthesis can be detected in oocytes labeled after defolliculation, either at 20°C or 35°C.

In about 30% of oocyte batches, the pattern of protein synthesis is completely different from that described above. There is little or no reduction in protein syn-

thesis on heat shock, and several unusual proteins are found in the oocyte itself (Fig. 7a). These are generally present at 20°C (track 1), but are further induced by heat shock (track 2). The major bands are due to proteins of about 60, 74, and 80 kD, but there is also a band of mobility very similar to *hap70*. Figure 7b shows part of a two-dimensional gel of such heat-shocked, defolliculated oocytes (cf. Fig. 7a, track 2). This profile is similar to that reported by Cuedon et al. (1985) in their study of the induction of the heat shock response of folliculated oocytes. The protein marked 3 is responsible for the 60 kD band in Figure 7a, and protein 4 is responsible for the 74 kD band. Proteins 5 and 6 contribute to the 70 kD band, but since they are not abundant or induced by heat shock, we believe that the major contribution on the 70 kD region of the one-dimensional gel is from another protein of 70 kD with an isoelectric point outside the range of this gel. Interestingly, proteins labeled 1 and 2 in Figures 5 and 6 are not detectable, as demonstrated in Figure 7c, which shows a mixture of the sample from Figure 7b with one from another experiment containing follicle cell-derived *hap70*. It is also clear from this figure that neither of the proteins 5 and 6 comigrates with bona fide *hap70* (marked H), so the 70,000 protein induced in this more unusual type of heat shock is not *hap70*.

We cannot explain the prominence of these unusual

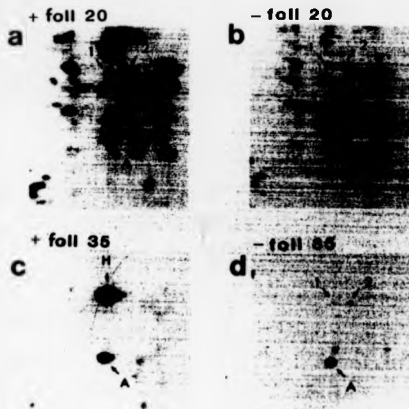


Figure 6. Two-dimensional analysis of protein synthesis in oocytes and follicle cells. Oocytes were incubated in [³⁵S]methionine for 2 hr either with their follicle cells intact (a and c) or after defolliculation (b and d). Samples a and b were labeled at 20°C and samples c and d at 35°C. (H) *hap70*, (A) actin. Proteins marked 1 and 2 are discussed in the text.

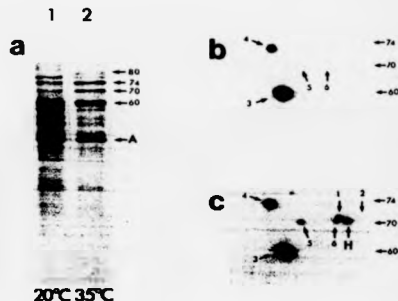


Figure 7. Abnormal pattern of heat shock protein synthesis. Oocytes were incubated in [35 S]methionine for 2 hr, defolliculated, and analyzed on 10% polyacrylamide gels. Track 1 shows oocytes labeled at 20°C, and track 2 shows oocytes labeled at 35°C. The heat-shocked, defolliculated oocytes were also analyzed on two-dimensional gels, both alone (b) and mixed with a sample of oocytes containing follicle cell-derived hsp70 (c). Estimated molecular weights of proteins are expressed in kilodaltons and indicated at the right-hand figure margins; the proteins marked 1-6 are discussed in the text. [H] hsp70, (A) actin.

60-, 70-, 74-, and 80-kD proteins in some batches of oocytes and not in others. One possible explanation is that they are modified forms of bona fide hsp70 (for example, the 70-kD proteins might be phosphorylated). This seems unlikely, however, since no intermediates or unmodified hsp70 are seen in defolliculated oocytes. So the most likely explanation is that they are entirely different proteins. Their expression could be due to a polymorphism in some aspect of control of the heat shock response, or it could be the response to an unidentified form of stress imposed on the frog or oocyte before the experiments were carried out. Because we rarely operate twice on the same frog, we cannot distinguish between these alternatives. The presence of these proteins does not alter our conclusion that hsp70 synthesis cannot be detected in oocytes, and emphasizes the need for two-dimensional gel analysis to distinguish bona fide hsp70 from proteins of similar molecular weights.

A possible reason for our failure to detect translation of hsp70 mRNA in oocytes could be that the protein is synthesized at a rate below the limit of detection by our experiments. We have attempted to address this question by examining oocyte polysomes for the presence or absence of hsp70 mRNA to assess its translation status at 35°C. Unfortunately, these experiments were inconclusive, due to the small amounts of hsp70 mRNA present. The question of translational "masking" of endogenous oocyte hsp70 transcripts therefore remains unresolved.

Expression from injected hsp70 genes

Any mechanism controlling selective translation of oogenic hsp70 mRNA could reasonably be expected to in-

volve specific sequence or structural elements within the mRNA. By reintroducing into the oocytes selectively mutated hsp70 transcripts, those regions of the mRNA that are involved could conceivably be identified. One strategy to achieve this would be to generate transcripts *in vivo* from injected genes. Arguably these transcripts might be regulated more appropriately than injected RNA. To test the feasibility of this approach, we have injected cloned hsp70 genes into oocytes.

Injected hsp70A genes are efficiently transcribed by oocytes maintained at normal temperatures. Up to 3.4 ng of hsp70A mRNA is accumulated by oocytes injected with 4 ng of hsp70A DNA during a 24-hr incubation at 20°C (data not shown). Heat shock for 2 hr at 35°C results in a detectable and reproducible increase in transcripts. The hsp70 mRNA found in injected oocytes is of the same length as hsp70 mRNA induced in follicle cells by heat shock (approximately 2.6 kb) and about 50% of transcripts are polyadenylated, as assessed by retention on oligo(dT)-cellulose columns (data not shown). Obviously, transcripts generated from hsp70 DNA would need to be located in the cytoplasm of the oocyte if they were to be of any use in analyzing translational control. Therefore, we investigated the accumulation and location of hsp70 mRNA in injected oocytes. Figure 8 (lanes 1-4) shows that the amount of hsp70 mRNA present in *Xenopus laevis* oocytes incubated at 20°C for various times after the injection of 4 ng of hsp70A DNA. Taking the amount of RNA present 24 hr after injection as 100%, surprisingly nearly 50% of this is produced within 2 hr and nearly 80% by 12 hr after injection. Eighty percent of the mRNA present after 24 hr at 20°C is present in the cytoplasm (Fig. 8, lanes 6 and 7). The 68%

increase in transcripts induced by 2 hr heat shock 24 hr after injection is confined to the nucleus and therefore heat shock has no effect on the amount of hap70 mRNA available in the cytoplasm [Fig. 8, lanes 4, 5, 8, and 9]. Using this approach an increase of around 1000-fold in hap70 mRNA content of the oocyte can be achieved, which, considering our difficulty in detecting hap70 protein synthesis from the endogenous RNA, allow us to investigate hap70 translational control in oocytes.

To investigate whether oocytes are capable of making hap70 protein if provided with more hap70 mRNA, we injected them with 4 ng/oocyte of hap70A DNA, allowed transcription to occur for 24 hr at 20°C, and then followed protein synthesis during a 2-hr period at 20°C or 35°C. Figure 9 shows that hap70 protein synthesis is easily detectable in injected oocytes that were defolliculated after labeling at 20°C and at 35°C [Fig. 9a,c]. This protein comigrates with follicle cell hap70 [data not shown]. In contrast, no hap70 can be detected in uninjected defolliculated oocytes [Fig. 9b,d].

These results suggest that masking of exogenous hap70 mRNA does not occur in *Xenopus* oocytes, and that hap70 protein can be made at 20°C as well as 35°C. *Drosophila* hap70 mRNA is translated efficiently in lysates of non-heat-shocked cells [Storti et al. 1980], so

there is no reason to believe that if the mRNA were present in a non-heat-shocked cell it would be translated less efficiently than other mRNA molecules. However, it remains possible that endogenous hap70 mRNA is masked in *Xenopus* oocytes, but the large numbers of hap70 RNA molecules transcribed from injected genes within 24 hr are not handled by the oocytes in the same way as the much smaller numbers of endogenous mRNA molecules made over a period of several months. The important result from this experiment is that hap70 mRNA transcribed from injected genes is preferentially translated at 35°C whereas the synthesis of other major proteins (such as actin) is barely detectable [Fig. 9c]. This cannot be explained simply as a consequence of induction of transcription during heat shock, as the RNase protection data shows that the same amount of RNA is present in the cytoplasm before and during a heat shock [Fig. 8]. Oocytes are therefore capable of translating hap70 mRNA appropriately. Since it has been demonstrated that injected *Drosophila* hap70 mRNA is translated in oocytes at 20°C but not 35°C [Bienz and Pelham 1982], our results support the view that the translation of hap70 mRNA in frog oocytes at 35°C is species specific. It is therefore not surprising that there is no homology between the 5' untranslated region of the *Drosophila* hap70 mRNA [Torok and Karch 1980], which contains translational control signals [McCarthy and Lindquist 1985; Klemenz et al. 1985], and that of *Xenopus* hap70 mRNA [Bienz 1984b].

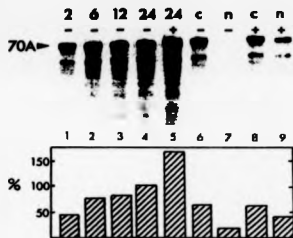


Figure 8. The accumulation and localization of transcripts from injected hap70A genes. Oocytes were injected with 4 ng of hap70A DNA per oocyte. Total RNA was extracted after 2, 4, 12, and 24 hr at 20°C (lanes 1, 2, 3, and 4, respectively) or after 24 hr at 20°C followed by a 2-hr heat shock at 35°C (lane 5). RNA was also extracted from the cytoplasm (c) and nucleus (n) of oocytes incubated for 24 hr at 20°C (lanes 6 and 7) or for 24 hr at 20°C followed by a 2-hr heat shock at 35°C (lanes 8 and 9). The position of hap70A transcript protected probe is indicated by the arrow. RNA equivalent to one oocyte was analyzed in each case. The lower panel indicates the relative amounts of hap70A transcripts for each sample, which were assessed by excising and counting the protected probe fragment. The level of transcripts accumulated after 24 hr at 20°C (lane 4) was taken as 100%.

Conclusions

Non-heat-shocked stage VI oocytes contain up to 13 pg of hap70A and B mRNA. These transcripts are accumulated during early oogenesis and stably retained throughout the remainder of oogenesis, maturation, ovulation, and early cleavage. No measurable increase or decrease of endogenous hap70 transcripts can be detected in the 24-hr period following heat shock. In contrast 70% of the hap70 transcripts induced in the surrounding follicle cells by heat shock disappear in the same post-heat shock period.

hap70 protein synthesis is not detectable in completely defolliculated oocytes, either before or during heat shock. The question of translational control of endogenous hap70 mRNA in oocytes therefore remains unresolved. Either these transcripts are not translated or they are translated at rates below the limit of detection by our analysis.

Injected hap70A genes are transcribed efficiently by oocytes at 20°C, leading to a rapid accumulation of nanogram quantities of hap70A mRNA. Cytoplasmic hap70 mRNA levels in DNA-injected oocytes are not affected by heat shock since induced transcripts remain within the nucleus during the course of the heat shock. hap70 protein synthesis is readily detectable in hap70 DNA-injected oocytes at both 20°C and 35°C, demonstrating that [1] hap70 mRNA can be translated by oocytes at 20°C and [2] hap70 mRNA is preferentially translated at 35°C.

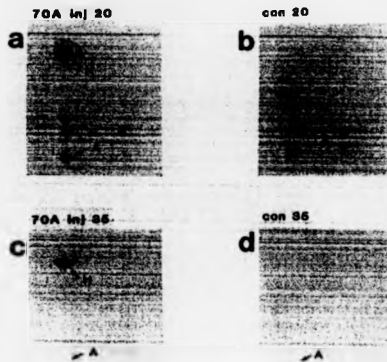


Figure 9. Expression of hap70 protein by oocytes injected with hap70 DNA. Oocytes were injected with 4 ng of hap70A DNA, incubated for 24 hr at 20°C, then labeled with [³⁵S]methionine at 20°C [a] or 35°C [c]. Uninjected control oocytes were also labeled at 20°C [b] or at 35°C [d]. All oocytes were defolliculated after labeling and before two-dimensional gel analysis. [H] hap70, [A] actin.

Although the question of translational "masking" of endogenous hap70 mRNA remains unsettled, these results allow the design of experiments to investigate the preferential translation of hap70 mRNA during heat shock. The heat shock response of *Xenopus* oocytes therefore provides a suitable system for studying this aspect of translational control.

Materials and methods

Oocytes and embryos

Oocytes were obtained from mature female frogs and maintained at 20°C in modified Barth's solution (MBS) as described by Coleman [1984]. Developmentally staged oocytes were prepared by incubating excised lobes of ovary in 2.5 mg/ml collagenase (Sigma, type IV), 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 15 mM Tris-HCl (pH 7.35), at 20°C for 2 hr (with gentle agitation) and manually sorting according to size and appearance [Dunson 1977]. Eggs and embryos were obtained as described by Cordon [1977] and staged according to Nieuwkoop and Faber [1967].

Defolliculation of oocytes

Oocytes were incubated in 0.25% collagenase (Sigma, type IV) in 88 mM NaCl, 1.0 mM KCl, 2.4 mM NaHCO₃, 15.0 mM Tris-HCl (pH 7.4) at room temperature for 10 min. The theca was then removed by manual dissection with fine watchmaker's forceps. After a further 5 min in collagenase, the oocytes were transferred to stripping solution (200 mM K aspartate, 20 mM KCl, 1 mM MgCl₂, 10 mM EGTA, 10 mM HEPES (pH 7.3), Sakmann et al. [1985]). This separated the vitelline layer from the

plasma membrane and allowed it to be removed by manual dissection. Somatic cells on the oocytes were visualized by staining in MBS containing 1 µg/ml Hoechst dye 33258. The number of follicle cells was estimated by photographing stained oocytes, counting the cells in a small circular area, and integrating this number over the surface area of the oocyte.

RNA extraction

Oocytes were either frozen rapidly on solid CO₂ and stored at -70°C or processed immediately. RNA was extracted by homogenization and incubation with SDS and proteinase K followed by repeated phenol extraction and precipitation with ethanol [Kreneman et al. 1978]. RNA was extracted in the same way from nuclei and cytoplasm separated by heating oocytes at 100°C for 3 min, chilling on ice, and manual dissection [Gazdov et al. 1984]. Approximately 5 µg of carrier RNA was added to nuclei before extraction. Any DNA recovered together with the RNA extracted from hap70 DNA-injected oocytes was not found to influence the RNase protection assay for hap70 transcripts, therefore additional DNase I digestion of nucleic acid preparations was not required.

DNA and sequencing

The hap70A and hap70B subclones pXLIQXP and pXLI16P were kindly provided by Dr. Mariann Bierns. Their construction from a *X. laevis* genomic library in λ2001 is described in Bierns [1984b]. The 484-bp AhaIII-HindIII fragment from pXLI16P, containing 5' untranslated and partial coding sequence for hap70B, was subcloned into M13 vectors mp18 and mp19 and sequenced by dideoxynucleotide chain termination [Sanger et al. 1977]. Comparison with the published hap70A sequence was performed using an IBM PC based Microgene package [Beckman].

RNase protection assay

The 484-bp *Ahoell-HindIII* fragment from pXLI6P was subcloned into pGEM-1 and transcribed *in vitro* using SP6 RNA polymerase and [γ - 32 P]UTP to produce a high-specific-activity antisense *hap70* RNA probe [Krieg and Melton 1987]. Full-length transcripts (506 nucleotides) were purified by electrophoresis and eluted from 4% acrylamide, 8 M urea gels and precipitated with ethanol. RNase protection assays were performed as described by Krieg and Melton [1987] using 4.5–22.5 μ g of total RNA (equivalent to 1–5 oocytes or embryos). Protected probe fragments were analyzed by electrophoresis on 4% acrylamide, 8 M urea gels (0.4 mm thickness), dried, and exposed to Fuji X-ray film with a Dupont intensifying screen. The amounts of *hap70* mRNA present in each sample were estimated by including a titration of known amounts of SP6-transcribed sense *hap70* RNA in each assay. Protected probe fragments were then cut out from dried gels and counted. In all but one experiment the assay gave a linear response over a range of 0–5000 pg of sense RNA.

Labeling of oocytes and electrophoresis

Oocytes were labeled in MBS containing [35]methionine at 1 mCi/ml for 2 hr. Heat-shocked oocytes were incubated for 15 min before addition of label to allow heat shock protein synthesis to begin. If oocytes had been defolliculated before labeling, they were labeled in MBS containing 5% dialyzed fetal calf serum. After incubation, the oocytes were washed in unlabeled MBS containing 1 mM methionine to prevent further incorporation of [35]methionine during processing. They were then defolliculated if necessary, and frozen on dry ice before homogenization in 0.1 M NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM Tris HCl (pH 7.6). Homogenates were centrifuged for 1 min in a microfuge to pellet the yolk platelets.

Samples were prepared for analysis on 10% SDS polyacrylamide gels [Laemmli 1970] as described by Colman [1984]. Typically, one-eighth of an oocyte was loaded per lane. For two-dimensional analysis [O'Farrell, 1975], homogenates were diluted with two volumes of lysis buffer. One-third of an oocyte was loaded onto each tube gel. Second-dimension slab gels were 10% acrylamide. After electrophoresis, gels were fixed, treated with EnHance [New England Nuclear], dried, and autoradiographed.

Acknowledgment

The authors are grateful to the British Cancer Research Campaign for support. Ann Horrell is in receipt of a Science and Engineering Research Council Studentship. We also greatly appreciate materials and advice from Dr. M. Bienz.

References

Bensaude, O., C. Rabreau, M. Morange, and F. Jacob. 1983. Heat shock proteins, first major products of zygotic gene activity in mouse embryos. *Nature* 305: 331–333.
 Bienz, M. 1984a. Developmental control of the heat shock response in *Xenopus*. *Proc. Natl. Acad. Sci.* 81: 3138–3142.
 ———. 1984b. *Xenopus hap70* genes are constitutively expressed in injected oocytes. *EMBO J.* 3: 2477–2483.
 ———. 1986. A CCAAT box confers cell-type-specific regulation on the *Xenopus hap70* gene in oocytes. *Cell* 44: 1037–1042.

Bienz, M., and J.B. Gordon. 1982. The heat-shock response in *Xenopus oocytes* is controlled at the translational level. *Cell* 29: 811–819.
 Bienz, M., and H.R.B. Pelham. 1982. Expression of a *Drosophila* *hap* in *Xenopus oocytes*: Conserved and divergent regulatory signals. *EMBO J.* 1: 1583–1588.
 Cabada, M.O., C. Darabrough, F.J. Ford, and P.C. Turner. 1977. Differential accumulation of two size classes of poly(A) associated with messenger RNA during oogenesis in *Xenopus laevis*. *Dev. Biol.* 57: 427–439.
 Callen, J.C., N. Dennebourg, and J.C. Mounolou. 1980. Kinetic analysis of entire oogenesis in *Xenopus laevis*. *Dev. Growth Differ.* 22: 831–840.
 Colman, A. 1984. Expression of exogenous DNA in *Xenopus oocytes*. In *Transcription and translation—A practical approach* [ed. B.D. Hames and S.J. Higgins], pp. 49–69. IRL Press, Oxford.
 Craig, E.A., T.D. Ingolia, and L.J. Manasek. 1983. Expression of *Drosophila* heat shock cognate genes during heat shock and development. *Dev. Biol.* 99: 418–426.
 Darabrough, C., and F.J. Ford. 1979. Turnover and processing of poly(A) in full-grown oocytes and during progesterone-induced maturation in *Xenopus laevis*. *Dev. Biol.* 71: 323–340.
 Di Domenico, B.J., G.E. Buzalsky, and S. Lindquist. 1982. The heat shock response is well-regulated at both the transcriptional and posttranscriptional levels. *Cell* 31: 593–603.
 Dumortier, J.N. 1973. Oogenesis in *Xenopus laevis*. I. Stages of oocyte development in laboratory maintained animals. *J. Morphol.* 136: 153–180.
 Georgiev, O., J. Moss, and M.L. Birnstiel. 1984. Processing and nucleocytoplasmic transport of histone gene transcripts. *Nucleic Acids Res.* 12: 8539.
 Glover, C.V.C. 1982. Heat shock induces rapid dephosphorylation of a ribosomal protein in *Drosophila*. *Proc. Natl. Acad. Sci.* 79: 1781–1785.
 Golden, L., U. Schrader, and M. Rosbach. 1980. Accumulation of individual pA⁺ RNAs during oogenesis of *Xenopus laevis*. *Cell* 22: 835–844.
 Gordon, G., D. Soria, J.P. Ebel, N. Belori, and P. Bony. 1985. Effect of diadenosine tetraphosphate aminoglycoside on heat shock protein synthesis in *Xenopus laevis* oocytes. *EMBO J.* 4: 3743–3749.
 Gordon, J.B. 1977. Egg cytoplasm and gene control in development. *Proc. R. Soc. B* 198: 211–247.
 Heikkilä, J.J., M. Kloc, J. Bury, G.A. Schultz, and L.W. Browder. 1985. Acquisition of the heat-shock response and thermotolerance during early development of *Xenopus laevis*. *Dev. Biol.* 107: 483–489.
 Hultmark, D., R. Klementz, and W.J. Gehring. 1986. Translational and transcriptional control elements in the untranslated leader of the heat-shock gene *hap22*. *Cell* 44: 429–438.
 King, M.L., and R. Davis. 1987. Do *Xenopus* oocytes have a heat shock response? *Dev. Biol.* 119: 532–539.
 Klementz, R., D. Hultmark, and W.J. Gehring. 1985. Selective translation of heat shock mRNA in *Drosophila melanogaster* depends on sequence information in the leader. *EMBO J.* 4: 2053–2060.
 Kressman, A., S.G. Clarkston, V. Pirrotta, and M.L. Birnstiel. 1978. Transcription of cloned tRNA gene fragments, and subfragments injected into the oocyte nucleus of *Xenopus laevis*. *Proc. Natl. Acad. Sci.* 75: 1176–1180.
 Krieg, P.A., and D.A. Melton. 1987. *Methods Enzymol.* (in press).
 Kurtz, S., J. Rossi, L. Petko, and S. Lindquist. 1986. An ancient developmental induction: Heat shock proteins induced in sporulation and oogenesis. *Science* 231: 1154–1157.

- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
- McGarry, T.J. and S. Lindquist. 1985. The preferential translation of *Drosophila* hsp70 mRNA requires sequences in the untranslated leader. *Cell* 43: 903-911.
- Mour, R.M. and J.C. Osborn. 1983. Somatic control of protein synthesis in mammalian oocytes during maturation. *Ciba Fond. Symp.* 98: 178-196.
- Nieuwhoop, P.D. and J. Faber. 1967. *Normal table of Xenopus laevis* [Davidin], 2nd ed. North-Holland Publishing Company, Amsterdam.
- O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250: 4007-4021.
- Parker, C.S. and J. Topol. 1984. A *Drosophila* RNA polymerase II transcription factor specific for the heat shock gene binds to the regulatory site of an hsp70 gene. *Cell* 37: 273-283.
- Pelham, H.R.B. 1982. A regulatory upstream promoter element in the *Drosophila* hsp70 heat shock gene. *Cell* 38: 417-528.
- . 1985. Activation of heat-shock genes in eukaryotes. *Trends Genet.* 1: 31-35.
- Ridlishough, G. and H.R.B. Pelham. 1986. Activation of the *Drosophila* hsp27 promoter by heat-shock and by ecdysone involves independent and remote regulatory sequences. *EMBO J.* 5: 1653-1658.
- Sakmann, B., C. Methfessel, M. Mishima, T. Takahashi, T. Takai, M. Kurasaki, K. Fukuda, and S. Numa. 1985. Role of acetylcholine receptor subunits in gating of the channel. *Nature* 318: 538-543.
- Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* 74: 5463-5467.
- Schlesinger, M.J., M. Ashburner, and A. Tissot. 1982. *Heat-shock from bacteria to man*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Storti, R.V., M.P. Scott, A. Rich, and M.L. Pardue. 1980. Translational control of protein synthesis in response to heat shock in *D. melanogaster* cells. *Cell* 22: 825-834.
- Torok, I. and F. Karch. 1980. Nucleotide sequences of heat shock activated genes in *Drosophila melanogaster*. I. Sequences in the region of the 5' and 3' ends of the hsp70 gene in the hybrid plasmid 56H8. *Nucleic Acids Res.* 8: 3105-3123.
- Wu, B.J. and R.I. Morimoto. 1986. Human hsp70 promoter contains a last two distinct regulatory domains. *Proc. Natl. Acad. Sci.* 83: 629-633.
- Zimmerman, J.L., W. Petri, and M. Meselson. 1983. Accumulation of a specific subset of *D. melanogaster* heat shock mRNAs in normal development without heat shock. *Cell* 32: 1161-1170.

THE BRITISH LIBRARY DOCUMENT SUPPLY CENTRE

TITLE

TRANSLATIONAL CONTROL OF THE HEAT SHOCK

RESPONSE IN XENOPUS OOCYTES

AUTHOR

Judith Ann Horrell

INSTITUTION
and DATE

University of Warwick
1988

Attention is drawn to the fact that the copyright of this thesis rests with its author.

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no information derived from it may be published without the author's prior written consent.

| | | | | | |
|-----|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 |
| cms | | | | | |

THE BRITISH LIBRARY
DOCUMENT SUPPLY CENTRE
Boston Spa, Wetherby
West Yorkshire
United Kingdom

20

REDUCTION X

CAMERA

2

D86759